Diacylglycerol lipase-dependent endocannabinoid signaling in neural stem cells

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A thesis for the degree of Doctor of Philosophy

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Abstract

The diacylglycerol lipases (DAGL α and β) synthesise 2-AG, the major endocannabinoid (eCB) in the developing and adult brain. 2-AG acts most prominently on the CB1 and CB2 receptors and growing evidence points to a role of eCB signaling in adult neurogenesis as well as a wide range of different physiological roles in the brain. The focus of this thesis is on DAGL driven eCB signaling in neural stem cells (NSC). A role of the eCB system has previously been established in migration, proliferation, and neurogenesis. Here we investigated DAGLα and eCB signaling in the context of differentiation.

The Cor-1 cells were adapted as a NSC model system and can be differentiated into glia and neurons. This differentiation was unaffected by CB signaling. While DAGLα expression is unaffected by glial differentiation, a rapid down regulation of DAGLα during neuronal differentiation was detected and we investigated the possibility of a specific degradation pathway being involved. DAGLα has a consensus motif for a putative destruction (d-) box that might target DAGL for ubiquitin-proteasome mediated degradation. No evidence was found to support that the d-box is involved in DAGLα degradation, however first indications towards degradation through the ubiquitin-proteasome pathway were identified.

What is upstream of eCB signaling in NSCs remains an open question. The most likely candidates are EGF, FGF-2 and insulin and their effect on eCB signaling was investigated. Evidence was found indicating that neither EGF nor FGFR signaling is upstream of eCB signaling in Cor-1 cells. Microarray analysis indicates a potential common signaling node between EGFR and eCB signaling. mTOR was investigated in this context, but while we show that mTOR appears to be downstream the EGFR, no indication was found that it is downstream of the eCB receptors. Further experiments are needed to determine the relationship between insulin and eCB signaling. It is essential to be able to measure DAGL activity, and a surrogate substrate DAGL activity assay was adapted to Cor-1 cells in order to address what is driving eCB signaling.

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Contributors

All studies described in this thesis were conducted by me unless otherwise stated.

Chapter III - Results 1:

The rapid reduction of DAGLα in Tuj-1 positive Cor-1 cells was first discovered by Dr Philipp Sütterlin (former Doherty lab member), but I carried out the described experiments.

Chapter IV - Results 2:

The bioinformatic analysis identifying the putative d-box in DAGLα was carried out by Dr Gareth Williams.

CHAPTER V - Results 3:

The presented microarrays were performed by Philip Sütterlin in collaboration with Wyeth research (now Pfizer) and the acquired data was pre-analysed by Dr Gareth Williams.

Publications arising from this thesis:

"Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice" (Gao et al., 2010)

"Down-regulation of diacylglycerol lipase-alpha during neural stem cell differentiation: identification of elements that regulate transcription" (Walker et al., 2010)

"The DAGLs: structure, regulation and roles in and beyond endocannabinoid signaling" (Reisenberg et al., in print)

"The molecular basis of the cooperation between EGF, FGF and eCB receptors in regulation of NSC function" (Sütterlin et al., submitted)

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Abbreviations

2-AG	2-Arachidonoylglycerol
Aa	Arachidonic acid
APC	Anaphase promoting complex
ATP	Adenosine-5'-triphosphate
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAM	Cell adhesion molecules
CB	Cannabinoid
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBD	Cannabidiol
CNS	Central nervous system
COX	Cyclooxygenase
C-terminus	Carboxyl terminus
DAG	Diacylglycerol
DAGK	Diacylglycerol kinase
DAGL	Diacylglycerol lipase
DAGLα orβ-V5	DAGLα or β with V5 tag
D-box	Destruction Box
DiFMU	6, 8-difluoro-4-methylumbelliferyl-octanoate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSE	Depolarisation induced suppression of excitation
DSI	Depolarisation induced suppression of inhibition
E	Embryonic day
eCB	Endocannabinoids

E. Coli	*Escherichia Coli*
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
EGF(R)	Epidermal growth factor (receptor)
FAC	Final assay concentration
FGF-2	Fibroblast growth factor 2
FGFR	Fibroblast growth factor receptor
FAAH	Fatty acid amide hydrolase
G 418	Geneticin
GABA	y-aminobutyric acid
GDP	Guanosine diphosphat
GFAP	Glial fibrillary acidic protein
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
IGFR	Insulin like growth factor 1 receptor
IGF-1	Insulin like growth factor 1
InsR	Insulin receptor
IP	Immunoprecipitation
kDa	kilo Dalton
KO	Knock out
LB	Luria bertani
LTD	Long term depression
LTP	Long term potentiation
M_1/M_3 R	Type 1/3 muscarinic acetylcholine receptor
MAGL	Monoacylglycerol lipase
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MDGL	Mono-and diglycerol lipase

mGluR	Metabotropic Glutamate receptor
(m) RNA	(Messenger) ribonucleic acid
MSE	Metabotropic suppression of excitation
MSI	Metabotropic suppression of inhibition
mTOR	Mammalian target of rapamycin
NAPE-PLD	N-acyl-phosphatidyledhanolamine-hydrolysing phospholipase D
NAT	N-acyltransferase
NSC	Neural stem cell
N-terminal	Amino terminus
OB	Olfactory bulb
p70	p70 S6 kinase
Pip_2	phosphatidylinositol 4,5-bisphosphate
PBS (T)	Phosphate buffered saline (with Tween20)
PD	Parkinson's disease
PDGF	Platelet derived growth factor
PFA	Para formaldehyde
PNS	Peripheral nervous system
PLC	Phospholipase C
PNP	4-nitrophenol butyrate
PPAR	Peroxisome proliferator activated receptor
RMS	Rostral migratory stream
RT	Room temperature
RTK	Receptor tyrosine kinase
TBS (T)	Tris buffered saline (with Tween20)
TaqMAN RT-PCR	Taqman reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SGZ	Subgranular zone
Shh	Sonic hedgehog homolog
Sp1	Specific protein 1
SVZ	Subventricular zone
SSI	Slow self inhibition
TGF	Tumor growth factor
THC	Tetrahydrocannabinol
THL	Tetrahydrolipstatin
TRPV1	Transient receptor potential vanilloid receptor
VEGF	Vascular endothelial growth factor
WT	Wild type

CHAPTER I: INTRODUCTION

1.1. Endocannabinoid signaling

1.1.1. The origins of endocannabinoid research

The cannabis plant has been used for medical and psychoactive reasons at least for several thousand years. It was discovered in the grave of a Chinese shaman and already contained its psychoactive substances (Russo et al., 2008). In ancient Europe, the cannabis plant was mostly used to make the fabric hemp and cordage until the knowledge about its psychoactive property was introduced to Europe by Napoleon in 1810 (Piomelli, 2003). The effects of cannabis consumption are remarkably diverse and include physiological as well as psychological effects. Cannabis has been used as medicine to manage pain and inflammation around the world across many centuries, but the psychoactive properties of the plant and a failure to isolate the active ingredients, limited its use. The challenge therefore is to understand the signaling of this system to allow separating the medically useful from the undesired effects.

1.1.2. Discovery of the eCB system

The effects caused by cannabis are mainly mediated by two substances, namely Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). THC is mainly known for its psychoactive properties, whereas CBD has been identified as the main non-psychoactive substance. While the study of other plant derived substances like morphine were already well advanced, the identification of the active chemical within the cannabis plant remained elusive until 1964/65, which is the time when the isolation and structural analysis of THC was first reported (Y. Gaoni, 1964; Mechoulam and Gaoni, 1965).

The creation of a selective THC analogue was key to the identification of the cannabinoid (CB)-sensitive brain regions (Melvin and Johnson, 1987; Devane et al., 1988) and led to the cloning of the first two cannabinoid receptors (CB1 and CB2 receptors) (Matsuda et al., 1990; Munro et al., 1993). In 1999, the first CB1 receptor knock out (KO) mouse was presented to the scientific world, with data showing that the mutant mice were largely unresponsive to CB drugs.

Furthermore, the CB1 receptor was demonstrated to be the mediator of analgesia, hypotension and hypothermia as well as having an involvement in the motivational aspects of opiates (Ledent et al., 1999). The creation of a CB2 receptor KO mouse followed shortly after (Buckley et al., 2000). Based on the high expression levels in the central nervous system (CNS), the CB1 receptor was thought to be having an impact here (Herkenham et al., 1991), whereas the CB2 receptor was associated with immune cells (Munro et al., 1993). The initial dogma, that the CB2 receptor does not have a role in the brain, has been revised based on more recent reports showing that the CB2 receptor is involved in NSC proliferation in cell culture as well as in the adult mouse (Molina-Holgado et al., 2007; Gao et al., 2010).

The CB1/2 receptors are cell membrane receptors belonging to the receptor family of G-protein coupled receptors. They display a protein sequence similarity of 44% (Munro et al., 1993) and, as typical for G-protein coupled receptors, they contain seven trans-membrane spanning domains. These domains are connected via three extra- and three intracellular loops. The N-terminus is located outside the cell, while the C-terminus is found within the cell (Pertwee, 2006). Upon stimulation G proteins couple to the receptor and are responsible for transmitting the signal further. It is thought that the G_i alpha subunit, which inhibits the production of cAMP from ATP mainly by inhibiting adenylate cyclase and increasing MAPK activity, is fundamentally important to this signaling cascade (Pertwee, 2006). cAMP is a second messenger and is involved in the regulation of iron as well as calcium channels. Furthermore, it interacts with a wealth of proteins such as PKA, PKC, ERK, p38 or Raf-1 (Pagotto et al., 2006). The downstream mechanisms of the CB1/2 receptors are an ever evolving field and is investigated in the context of different cells (e.g. Brighton et al., 2011; Merighi et al., 2012). A recent study investigated the signaling of the human CB1 receptor splice variants CB1a and CB1b receptors and compared them to the rat CB1 receptor. They discovered that in hippocampal neurons the human CB1 receptors inhibited synaptic transmission less compared to the rat CB1 receptor. This might indicate different downstream signaling mechanisms between the rodent and the human CB1 receptors and

underlines the importance of investigating the human CB1 receptor (Straiker et al., 2012).

The hunt for the endogenous CB ligand(s) has identified the lipid anandamide (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) as putative endocannabionids (eCBs) (Mechoulam et al., 1995; Sugiura et al., 1995). 2-AG can cause THC-typical effects (Mechoulam et al., 1995) and has been argued to be the true ligand for the CB1/CB2 receptors based on the observation that it is a full agonist (Sugiura et al., 1999). The paper revealed that 2-AG gave a maximal response, which exceeded all other examined compounds and shows activity from as little as 0.3nM. Anandamide was explored and categorized as a partial agonist (Sugiura et al., 1999).

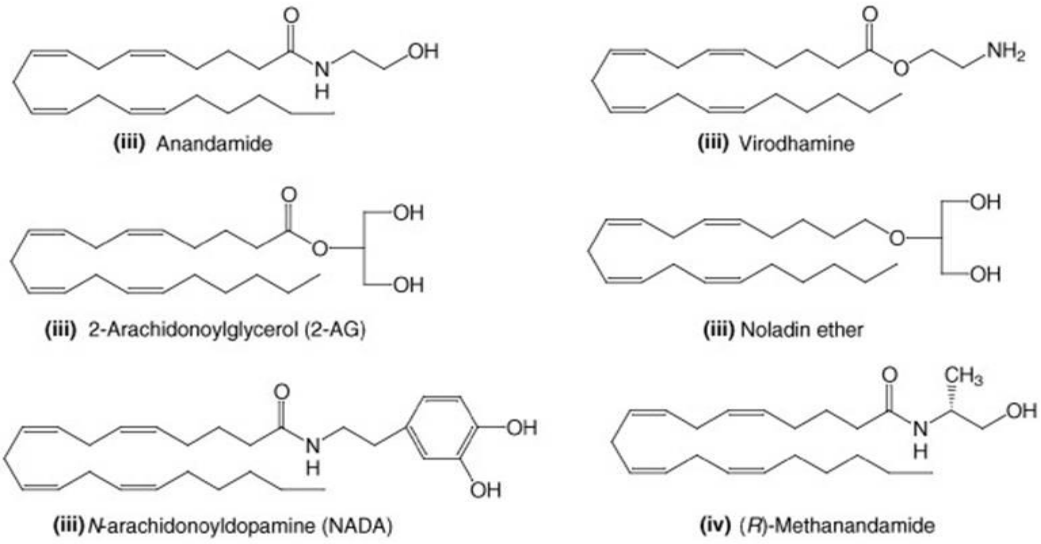


Figure 1.1: Structure of eCBs

Figure adapted from (Matias and Di Marzo, 2007)

Additional receptors and putative eCBs have been discovered. It has been suggested that the G-protein coupled receptor 55 (GPR55) (Baker et al., 2006), the transient receptor potential of vannilloid type-1 (TRPV1) (Zygmunt et al., 1999; Smart et al., 2000) and the peroxisome proliferators activated receptor (PPAR)α (Burstein et al., 2004; Burstein, 2005; Rockwell et al., 2006) are all part of the eCB

system. However, the involvement of GPR55 is largely based on the observation that pharmacological agents that act on CB1 and/or CB2 receptors can act on GRP55 receptors, and therefore whilst a pharmacologic interaction is established, the physiological response requires further study. The more recently discovered CB ligands, noladin ether (Mechoulam et al., 1998; Fezza et al., 2002), virodhamine (Porter et al., 2002) and N-arachidonoyldopamine (Bisogno et al., 2000; Hu et al., 2009) have been shown to be able to activate CB1 and/or CB2 receptors in pharmacological assays, but there is as yet no evidence that they do so in a physiological context. If one sticks to a more rigid set of criteria that includes evidence of physiological effects, the CB1 and CB2 receptors together with anandamide and 2-AG can perhaps be best considered as constituting the core identified ligands and receptors of the eCB signaling system.

In order to establish a more comprehensive understanding of eCB signaling, it is crucial to understand how and where the putative eCBs are synthesized and broken down, as this will dictate whether they have an opportunity to serve as ligands for the CB1/CB2 receptors. The diacylglycerol lipase (DAGL) homologues α and β were shown to synthesize 2-AG from diacylglycerol (DAG) (Bisogno et al., 2003). 2-AG levels in the brain are decreased by 80% in DAGLα KO mice and drop by 50% in DAGLβ KO mice. Furthermore, a drastic reduction of arachidonic acid as well as anandamide was observed (Gao et al., 2010). How this is linked to the more canonical anandamide biosynthesis from membrane phospholipids via N-acyltransferase (NAT) and N-Acyl-Phosphatidyledhanolamine-Hydrolysing Phospholipase D (NAPE-PLD) is not fully understood yet. The creation of monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) KO animals allowed for clear answers to the question as to how the eCBs are further metabolised. Anandamide seems to be predominantly degraded by FAAH (Cravatt et al., 2001), while 2-AG can be broken down to arachidonic acid by MAGL (Schlosburg et al., 2010), a process which links the eCB signaling to prostaglandin signals (Nomura et al., 2011). While this seems to be the main degradation pathway for 2-AG, metabolism/degradation via other pathways has been described by FAAH or cyclooxygenase (COX)-2 (Sugiura et al., 2002). Together this shows the

complexity of eCB signaling as well as the interaction with a multitude of other signaling pathways.

1.1.3. Identification and characterization of DAGL signaling

The two human DAGL isoforms α and β were first cloned in 2003 and identified as the enzymes to synthesize 2-AG from DAG (Bisogno et al., 2003). 2-AG has been identified to be the key eCB in many physiological contexts such as axonal growth and guidance, as a retrograde messenger at synapses and in adult neurogenesis all of which are discussed below.

DAGL activity was first studied in the context of arachidonic acid released from platelets. The observed lipid release involves DAGL as well as phospholipase C (PLC) activity and could be stimulated with calcium (Bell et al., 1979). Metabolism of DAG to arachidonic acid was identified as a two step mechanism shortly afterwards. First, DAG is hydrolysed to 2-AG at the *sn*-1 position. Second, MAGL metabolizes 2-AG further to arachidonic acid (Prescott and Majerus, 1983). Alongside MAGL, DAGL was first attempted to be purified from the bovine brain. Its activity was identified to be adenosine triphosphate (ATP) dependent displaying a more potent inhibitory activity compared to adenosine diphosphate (ADP) or adenosinmonophosphate (AMP), whilst cyclic adenosine monophosphate (cAMP) marginally increased activity (Farooqui et al., 1984). It was purified from bovine brain microsomes and showed a molecular weight of 27 kDa when run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). At low concentrations, neither calcium nor magnesium had an effect on enzyme activity but a threefold increase was observed after phosphorylation by a cAMP-dependent protein kinase (Rosenberger et al., 2007). This became relevant to neuroscience, when the DAGL-MAGL pathway was demonstrated to release arachidonic acid from cultured dorsal root ganglion neurons (Allen et al., 1992).

Especially earlier DAGL studies relied heavily on the semi-selective DAGL inhibitor RHC80267 (Sutherland and Amin, 1982), which also displays a considerable efficacy towards other serine lipases. When comparing another DAGL inhibitor tetrahydrolipstatin (THL) with RHC80267, a minimal overlap of target profiles was

seen; indicating that an effect seen by both drugs has a high likelihood to be a DAGL-dependent event. In the case of a known CB1/CB2 receptor dependent event, RHC80267 remains helpful regarding the differentiation between a 2-AG and anandamide mediated event (Hoover et al., 2008). THL, also more commonly known as orlistat is known for its inhibition of pancreatic lipase. This lipase is well studied in the context of weight loss (Hadvary et al., 1991) and THL is currently sold as an over the counter weight loss aid in the UK. A THL analogue (OMDM-188) promises to be the next generation of DAGL inhibitors due to its high specificity (Ortar et al., 2008).

1.1.4. Cloning of DAGLα and DAGLβ and creation of KO mice

The human DAGLs were identified by comparing the DNA sequence of a known mono-and diglycerol lipase (MDGL) from *Penicillium camembertii* to the human genome (Yamaguchi et al., 1991; Bisogno et al., 2003). Peniccillia are known to be good producers of lipases and are extensively studied for that reason. MDGL and other lipases have a wide range of industrial potential (Ning Li, 2010). MDGL was not able to use triglycerols as a substrate and displayed a unique specificity for acylglycerols (Yamaguchi et al., 1991; Bisogno et al., 2003). The catalytic amino acids of this 276 amino acid containing protein were identified by site directed mutagenesis and Yamaguchi et al. developed an efficient expression system after another attempt in *Sacchraromyces cerevisiae* failed based on high glycosylation levels (Yamaguchi et al., 1992; Yamaguchi et al., 1997). Mono- and diacylglycerols are food emulsifiers and are also used in cosmetics and pharmaceuticals and therefore are of interest to several industries. Furthermore, they are building blocks in the synthesis of glycolipids and phospholipids, as well as some prodrugs including one Parkinson's disease drug (Ning Li, 2010).

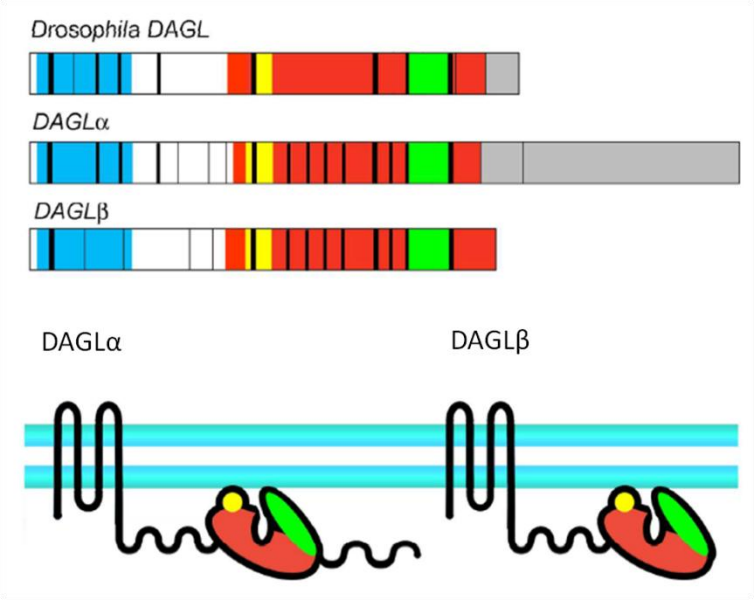


Figure 1.2: Exon structure and overall domain structure of DAGLα and β

The exon structure of the drosophila DAGL and the vertebrate DAGLα/β are shown as a linear schematic. Vertical lines indicate the exon boundary locations with bold lines indicating conservation between the three enzymes. The 4TM region is shown in blue, the catalytic domain in red, and the tail region in green. A cysteine rich sequence is highlighted in yellow and the regulatory loop, encoded by a single exon, is shown in green. A schematic representation of the domain structure is shown below the linear schematic and the same colour scheme was used as described above (Reisenberg et al., In Print).

Human DAGLα is the larger of the two enzymes, containing 1042 amino acids (120 kDa) compared to 672 amino acids (70 kDa) of DAGLβ. The main difference between DAGLα and β can be found after the catalytic domain in the tail region. Both proteins have a lipase-3 motif, a serine lipase motif and a four membrane-spanning domain with the catalytic domain as well as the N-Terminus located within the cell. The two enzymes display a remarkable homology, with a very high conservation between man and mouse (Bisogno et al., 2003).

During development, the DAGLs are expressed in the axonal tracts, while they are restricted to dendritic fields in the adults. This location change goes hand in hand with a switch in functionality between development and adulthood. DAGLα is necessary for axonal growth and guidance during development, while in the adult it is involved in synaptic transmission (see below for more information). TaqMan RT-PCR was used to establish the DAGLα transcript levels in different mouse and

human tissue. It most prominently is expressed in the brain and spinal cord of mice. In the human its expression peaks in the brain and pancreas (Bisogno et al., 2003), where DAGL activity has been reported to be involved in amylase secretion (Hou et al., 1997).

Given the pharmacological limitations of the available drugs, it was essential to create DAGL KO mice to test their function in eCB signaling. Two separate models were published within one day from each other (Gao et al., 2010; Tanimura et al., 2010). In DAGLα KO mouse brains and the spinal cord, an 80% reduction in 2-AG levels was observed while a 50% reduction was seen in DAGLβ KO mouse brains. However, DAGLβ seems to have a significant role in the liver, as 2-AG levels are reduced by 90% in DAGLβ KO mice. Arachidonic acid levels changed in parallel to 2-AG, displaying a DAGL dependency of its steady state level. Furthermore, anandamide levels were also reduced (Gao et al., 2010). This most probably relates to a complex interplay between the steady-state levels of various arachidonate containing lipids rather than a direct role of the DAGLs in the "on-demand" synthesis of anandamide (reviewed in Di Marzo, 2011). Despite many efforts and candidate pathways, the pathways regulating the "on-demand" synthesis of anandamide are still not fully understood (Alger, 2012). Nevertheless, the DAGL KO mice provide us with an excellent tool to establish the role of these enzymes in eCB pathways. As will be discussed below, they have revealed an essential role for DAGLα in retrograde synaptic signaling throughout the brain, and a role for DAGLα and DAGLβ in the control of adult neurogenesis.

1.1.5. The influence of DAGL-dependent eCB signaling on axonal growth and guidance

During development a variety of different processes have to take place to create a functional brain. These processes include differentiation, migration, synapse formation and axonal growth and guidance. Here, we will describe the role the eCB system plays in axonal growth and guidance. This is the process by which neurons extend their axons with the help of different guidance clues to find and innervate their appropriate targets.

Cell adhesion molecules (CAM) such as neural CAM (NCAM), N-cadherin and L1 are most prominently known for their adhesive properties, but their ability to signal via second messenger cascades is also required to promote a variety of biological processes (Cavallaro and Dejana, 2011). They have been shown to act as surrogate ligands for the FGFR (fibroblast growth factor receptor) and by doing so trigger calcium influx into the growth cone. This influx of calcium is necessary and sufficient to promote axonal growth and guidance. FGFR activation stimulates a PLCγ-dependent synthesises of DAG, the substrate of the DAGLs. Inhibition of DAGL can furthermore abolish neurite outgrowth stimulated by the above CAMs. This mechanism was shown to operate upstream of calcium influx into growth cones via N and L-type calcium channels (Williams et al., 1994b; Williams et al., 1994a; Hall et al., 1996; Walsh and Doherty, 1997). FGF (fibroblast growth factor) can also stimulate neurite outgrowth through the same activation of PLCγ as well as DAGL to stimulate calcium influx into growth cones (Williams et al., 1994b).

DAGLα and β are coexpressed in the axonal tracts during development (Bisogno et al., 2003). They are expressed in the same growth cones as the CB1 receptor at this stage of development (Berghuis et al., 2007) and CB1 receptor function has been identified to be downstream of N-cadherin and FGF2 signaling, but upstream of the observed calcium influx (Williams et al., 2003). The DAGLs generate 2-AG, which has also been shown to act as an autocrine signal *in vivo* in pyramidal cell axons. eCB signaling is required for developing pyramidal cells for axonal elongation and fasciculation and moreover CB1 receptor null mice , and pyramidal cell specific conditional CB1 receptor deficient mice furthermore display impairments in axonal fasciculation (Mulder et al., 2008). The CB1 receptor is also abundantly present in axonal growth cones of γ-aminobutyric acid (GABA)ergic interneuron during rodent cortex development. The Harkany lab also established that target selection is impaired when the CB1 receptor is specifically deleded from GABAergic interneurons (Berghuis et al., 2007). The 2-AG degrading enzyme MAGL appears to be used to fine tune 2-AG signaling in the context of axonal growth and guidance. MAGL is expressed during development in the axonal tracts but is excluded from

the growth cone. This expression pattern helps 2-AG mediated axonal growth (Keimpema et al., 2010).

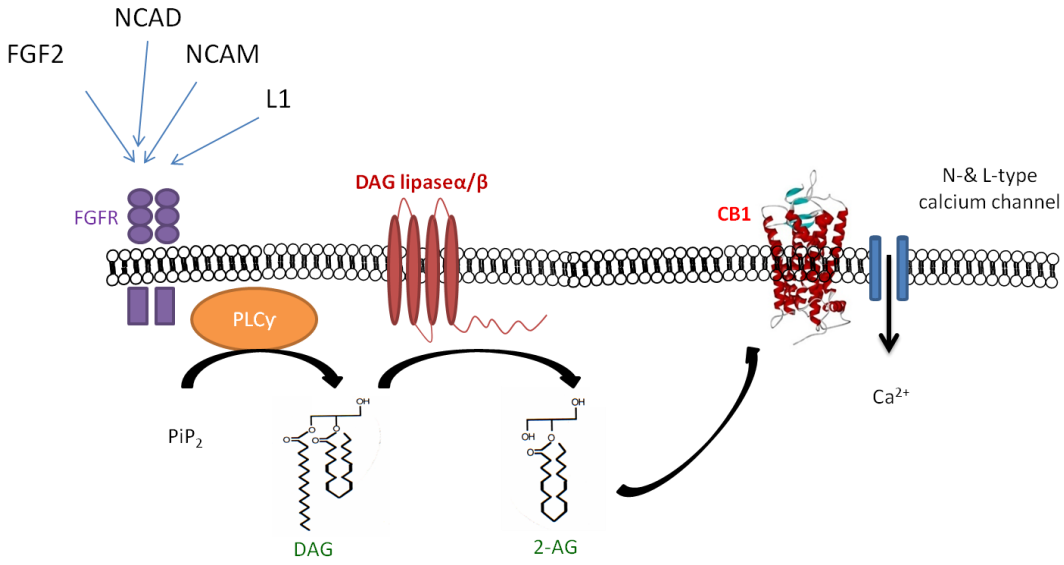


Figure 1.3: The role of the eCB system for axonal growth and guidance

This is a simplified model of eCB signaling in the context of axonal guidance. The FGFR is activated and stimulates PLCγ, which generates 2-AG from DAG. 2-AG then activates the CB1 receptor in an autocrine fashion which in turn leads to an internal calcium increase via L-& N-type calcium channels.

The exact mechanism of how DAGL/eCB signaling influences calcium influx is not fully understood and seems to be context dependent. While some research points towards a G_i-dependent mechanism, based on the observation that calcium influx can be inhibited with pertussis toxin (Sugiura et al., 1996), others found that CB1 receptor agonist dependent calcium influx was not inhibited by the use of pertussis toxin, indicating a G_i-independent mechanism (Rubovitch et al., 2002). Together this data provides evidence for a CAM/FGFR dependent calcium influx into growth cones via a DAGL dependent eCB signaling pathway, but further research is needed to understand the exact mechanism.

Whether eCB signaling promotes or inhibits axonal growth is context dependent. FGF-2 and CAM signaling have been demonstrated to promote axonal growth and guidance via a DAGL dependent eCB cascade (Williams et al., 2003; Keimpema et al., 2010). However, in other scenarios growth cone collapse was triggered by CB1

receptor agonist exposure (Berghuis et al., 2007; Argaw et al., 2011). The upstream guidance clues such as FGF2 also have the ability to promote or inhibit axonal growth. Cerebellar granule cells which are grown on laminin and 3T3 fibroblasts show increased neurite outgrowth in the presence of FGF-2 concentrations. The opposite is seen using the same experimental setup where the cells are grown on an astrocyte monolayer (Williams et al., 1995). These results indicate that neurite outgrowth is highly dependent on the context the cells are exposed to.

Given the importance of the eCB system for axonal growth and guidance it is surprising that the CB1 receptor KO mice, as well as the DAGLα KO mice, were overall healthy without any obvious serious defects in the brain (Ledent et al., 1999; Gao et al., 2010). One answer to this might be the high redundancy of ligands and receptors. In the absence of one of the ligands or receptors, the cells might be able to compensate by signaling via an alternative signaling route. This might mean, that a multitude of genes have to be knocked out before any effect can be seen. Another noteworthy aspect is to consider the context the signaling pathway is researched in. Older CB1 receptor KO mice have shown an increased decline in spatial learning as well as a neuronal loss in the hippocampus (Albayram et al., 2011). Younger CB1 receptor KO mice are relatively normal, and the full effect can only be seen in older animals suggesting that the full effect of the receptors loss can only be observed later on in life of the animals. The full effect of the CB1 receptor KO in younger mice therefore is masked and could have been overlooked without the use of the older mice. We also have to remind ourselves of the complexity of the human brain, where even a small miswiring might lead to diseases as severe as Schizophrenia (Rapoport et al., 2005).

1.1.6. The role of DAGL in synaptic plasticity

It often occurs that the same pathways which regulate axonal growth and guidance also regulate synapse plasticity (Frank and Tsai, 2009). This also holds true for DAGL dependent eCB signaling. Synaptic plasticity is the change in connectivity between neurons, which depends on experience and is thought to be the underlying mechanism for learning and memory (J. C. ECCLES, 1951). A key aspect of trans-synaptic regulation is retrograde signaling, which describes the process by

which the molecule travels across the synaptic cleft from the postsynapse back to the presynapse. Different molecules have been suggested to act as retrograde messengers including carbon monoxide (Alkadhi et al., 2001), platelet derived factor (Kato et al., 1994), arachidonic acid (Malenka and Bear, 2004), and the brain derived neurotrophic factor (BDNF) (reviewed Regehr et al., 2009). The eCBs are also a key class of retrograde messengers in the mammalian brain (discussed below).

There are different forms of retrograde neuronal plasticity, and overall they are generally distinguished by the nature of the stimuli which induce them. As mentioned above, there is a variety of different stimuli, which are involved in retrograde signaling. DSE and DSI are the depolarization-induced suppression of excitation or inhibition respectively (Straiker and Mackie, 2005). Both DSI and DSE occur throughout different brain regions, and DSE has been reported in the hippocampus, hypothalamus and the striatum, while DSI was demonstrated in the neocortex, cerebellum as well as the substantia nigra (reviewed in Hashimotodani et al., 2007b). Other forms of stimulation are the high-frequent stimulation which increases synaptic strength (long term potentiating; LTP) and a low frequency stimulation causing synaptic weakening (long term depression, LTD). The role of the eCB system for these forms of synapse plasticity has been described in considerable detail elsewhere (Hashimotodani et al., 2007b; Kano et al., 2009). Both forms of synaptic plasticity can be potentially found at every excitatory synapse of the mammalian brain (Malenka and Bear, 2004). Further mechanisms are metabotropic suppression of inhibition/excitation (MSI/MSE), which describes a form of neuronal plasticity via metabotropic glutamate receptors (mGluR). In neocortical interneurons, a 2-AG dependent CB1 receptor activation within the same cell has been described and named slow self inhibition (SSI) (Marinelli et al., 2008).

The components of the eCB system are ideally located for their role in synaptic plasticity driven by retrograde signaling. Generally speaking, in the context of synapse plasticity 2-AG is thought to be synthesized by DAGL in dendritic spines of excitatory neurons and is discharged into the synaptic cleft where it mainly

interacts with CB1 receptors located at the pre-synaptic terminal. 2-AG signaling is then terminated by MAGL, which is also often expressed presynaptically (reviewed in Oudin et al., 2011a). A fundamental switch in DAGL expression occurs between development and the adult brain. While DAGLα is expressed in the axonal tracts during the development, it is excluded from the same tracts in the adult and becomes highly enriched in dendrites (Bisogno et al., 2003). This switch in location allows for a switch in function. A very comprehensive study investigating the expression of DAGLα in the rat brain was carried out and identified a widespread distribution of the enzyme in tune with its role in synapse plasticity throughout the brain (Suarez et al., 2011). CB1 receptor is mainly expressed in the same brain regions (Herkenham et al., 1991), ensuring that 2-AG is highly accessible to the receptor. To regulate 2-AG signaling, MAGL was originally thought to be expressed mainly in the presynaptic terminals of neurons capable of eCB signaling (Gulyas et al., 2004), but it is also now know to be expressed in surrounding synapses and astrocytes cells as well (Uchigashima et al., 2011), with expression in the astrocytes also regulating the duration of the retrograde signal. A more detailed description of eCB expression in this context can be found elsewhere (Oudin et al., 2011a).

Hippocampal DSI and cerebellar DSE and DSI have been shown to be mediated through a depolarisation induced eCB release from the dendritic spines which inhibit the release of neurotransmitter by interacting with presynaptic CB1 receptors (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson et al., 2001). The inhibition of the neurotransmitter release is usually a consequence of brief CB1 receptor stimulation which induces an inhibition of voltage-gated calcium channels and other studies have reported an increase in potassium conductance (reviewed in Alger and Kim, 2011).

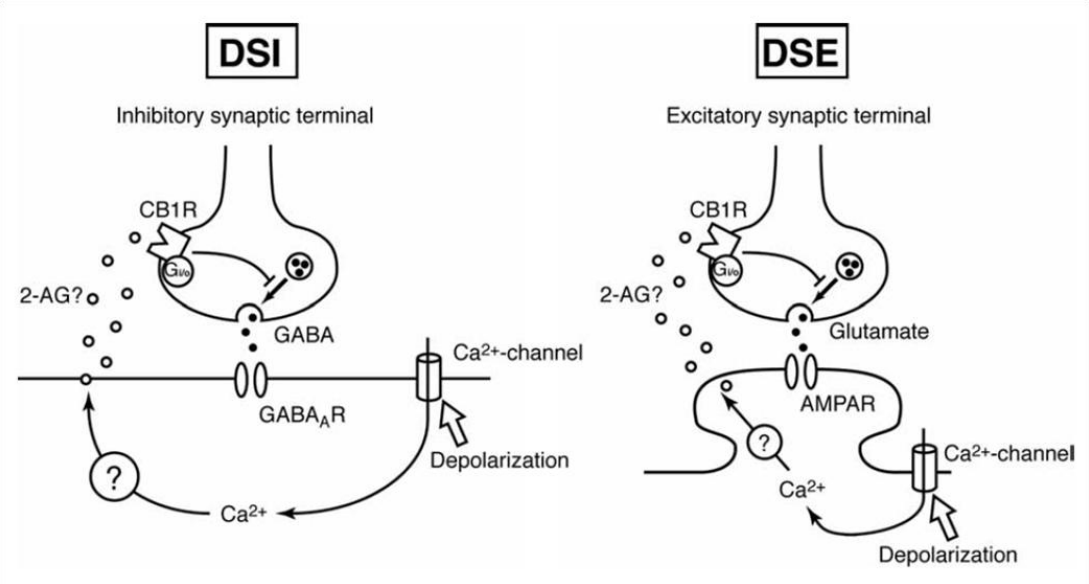


Figure 1.4: eCB mediated DSI and DSE

The voltage-gated calcium influx is induced by postsynaptic depolarization. This leads to an increase in intracellular calcium concentration and metabolism of eCBs, most likely 2-AG. 2-AG is then released into the synaptic cleft and activates the presynaptically located CB1 receptor, and this leads to the suppression of the neurotransmitters GABA (DSI; A) and glutamate (DSE; B) (figure taken from Hashimotodani et al., 2007b).

A form of eCB release which is receptor driven has also been reported. The activation of metabotropic glutamate and/or acetylcholine receptors have been reported to trigger the previously described suppression of excitatory or inhibitory inputs (Maejima et al., 2001; Kim et al., 2002). This has been reported to be possible without calcium elevation. It is thought to be mediated by mGluR type 1 or 3 and muscarinic acetylcholine receptors type 1 and 3 (M_1/M_3 receptors) via G-proteins stimulation of certain PLCβs (Hashimotodani et al., 2007b). This in turn leads to an increase in DAG levels which can be hydrolysed by DAGL to 2-AG. A synergistic mechanism between receptor activation and calcium elevation has also been reported and seems to be particularly effective. While it is possible that the two mechanisms work independently, it is thought that both mechanisms act by stimulating PLCβ (Maejima et al., 2001; Ohno-Shosaku et al., 2005).

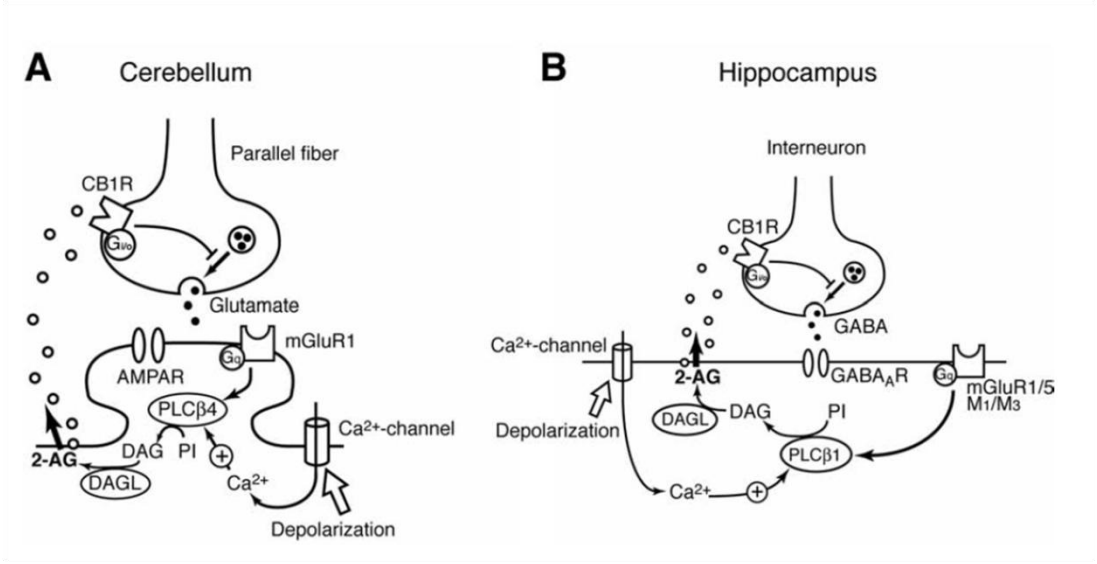


Figure 1.5: Heterosynaptic and homosynaptic regulation of neurotransmitter release though eCB signaling

The model which can be seen above displays the receptor driven eCB release dependent and independent of calcium influx into the cell. This mechanism is dependent on PLCβ stimulation by muscarinic acetylcholine receptors (M_1/M_3) or mGluR 1/5. The effect can be enhanced by an additional increase in the intracellular calcium concentration (figure taken from Hashimotodani et al., 2007b).

Using different approaches like the use of DAGL inhibitors or inhibition of 2-AG breakdown, 2-AG has been implied to be the relevant eCB in the context of DSE and DSI (Chevaleyre and Castillo, 2003; Kim and Alger, 2004; Chevaleyre et al., 2006; Hashimotodani et al., 2007a; Uchigashima et al., 2007; Pan et al., 2009). Nonetheless, the theory that 2-AG was responsible for retrograde signaling was not universally accepted. In the hippocampus, some groups found that the DAGL inhibitor RHC80267 failed to inhibit DSI at inhibitory synapses (Chevaleyre and Castillo, 2003; Edwards et al., 2006), while in the cerebellum it had no effect on DSE (Safo and Regehr, 2005). Using another DAGL inhibitor (THL), the role of DAGL in hippocampal DSI and cerebellar DSE was demonstrated. One possible explanation for these different findings is the poor selectivity of DAGL inhibitors. Hopes were high for the new DAGL inhibitor OMDM-188 (Ortar et al., 2008), which is more active but whose selectivity against a wide range of lipases and other targets has yet to be established. It was shown, that OMDM-188 and RHC-80267 did not affect

hippocampal DSI, while THL gave an application dependent result regarding its influence on DSI. All three inhibitors showed that self-inhibition in neocortical interneurons was blocked (Min et al., 2010b).

A plausible explanation for the seemingly contradicting results would be that under certain circumstances, eCBs might be stored presynaptically rather than being produced on demand (Min et al., 2010a), however there is as yet no direct evidence to support this. Furthermore, OMDM-188 and other DAGL drugs are not able to distinguish between the two DAGL isoforms, which makes the recently produced DAGLα and β KO mice extremely valuable tools for critically testing their requirement for DSI/DSE. With the help of the KO mice it was shown that DAGLα, but not DAGLβ, is required for DSI in hippocampal CA1 neurons (Gao et al., 2010). DSE and DSI were also demonstrated to be absent from purkinje cells, and interneurons in an independently generated DAGLα KO mouse model while calcium levels were comparable to wt mice (Tanimura et al., 2010). Furthermore, it was shown that in the prefrontal cortex, DAGLα (and not DAGLβ) displays a role in suppression of inhibitory transmission (Yoshino et al., 2011). These and other experiments suggested 2-AG as the chief eCB involved in synaptic plasticity. Anandamide is the other heavily studied eCB, but it is thought to have limited effect on altered synapse transmission via retrograde signaling. Most convincingly DSE/DSI were unaltered by changes in FAAH levels, the main anandamide degrading enzyme (reviewed in Alger and Kim, 2011). The DAGLα/β KO mice were thought to reveal a final answer to this question, but surprisingly, the DAGLs also displayed a considerable influence on basal anandamide levels, even though to a much smaller degree than 2-AG (Gao et al., 2010; Tanimura et al., 2010). A role of anamdamide in synapse transmission regulation cannot be ruled out, but the abundant pharmacological data points to 2-AG as the main eCB involved in synaptic plasticity (Pan et al., 2009; Straiker et al., 2009; Alger and Kim, 2011).

1.2. Neurogenesis

The CNS of mammals and advanced vertebrates is complete after the stages of developmental neurogenesis. The brain however displays many forms of plasticity to adapt to new environments and situations. Adult neurogenesis is a form of

cellular plasticity and has been first suggested over 50 years ago. Despite a struggle to be widely accepted at first it is now firmly established. A nice overview about the initial findings and the history of neurogenesis research can be found elsewhere (Chojnacki et al., 2009).

In the adult brain, neurogenesis is well documented in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus (Altman, 1962, 1969; Lois and Alvarez-Buylla, 1994). Adult neurogenesis has been linked to learning and memory and disruptions in the process have been related to a range of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.

1.2.1. Neural stem cells and the adult neurogenic niches

The SVZ is located in the lateral wall of the ventricle and is the largest and arguably the best studied germinal region in mammalian adult brains. It provides the olfactory bulb (OB) with new neurons via the rostral migratory stream (RMS) as well as the prefrontal cortex in humans after birth (Sanai et al., 2011). The supply of new neurons to the OB appears to be important for some aspects of olfactory function (Gheusi et al., 2000; Breton-Provencher et al., 2009). Furthermore, neuroblasts from this area have the ability to migrate to injured brain sites, where they might limit damage and/or contribute to the recovery of function (Arvidsson et al., 2002; Goings et al., 2004; Zhang et al., 2004). The importance and functionality of the new neurons was critically discussed (Kempermann et al., 2004) and further research is needed, but overall it is thought that regulation of adult neurogenesis could offer a wide range of therapeutic possibilities.

The SVZ harbours 4 main cell types which were characterized at the ultrastructural level. These cells are neuroblasts (Type A-cells), SVZ astrocytes (Type B-cells), immature precursor cells/transient amplifying cells (Type C-cells) as well as ependymal cells (Doetsch et al., 1997). A layer of ependymal cells separates the lateral ventricle and the SVZ, even though occasionally B-cells are in direct contact with the ventricle via a single process (Doetsch et al., 1999a). B-cells can also connect to blood vessels via an extension of basal processes. This enables them to

receive signals from the blood and the cerebrospinal fluid (Tavazoie et al., 2008). In this niche, B-cells give rise to the transient amplifying Type C-cells, which differentiate into migratory neuroblasts that travel along the RMS to the OB and eventually differentiate into neurons (Doetsch et al., 1999b; Doetsch, 2003a). The identity of the true NSC has been discussed excessively and is still debated (Chojnacki et al., 2009). While some arguments were found favouring ependymal cells (Johansson et al., 1999) others doubted the neurogenic potential of these cells (Capela and Temple, 2002; Spassky et al., 2005). SVZ astrocytes were suggested as an alternative to be the true NSCs (Doetsch, 2003a). At present, the astrocytic marker GFAP (glial fibrillary acidic protein) is often taken as a NSCs marker. It has been argued though, that the expression of GFAP is neither sufficient nor necessary (Chojnacki et al., 2009).

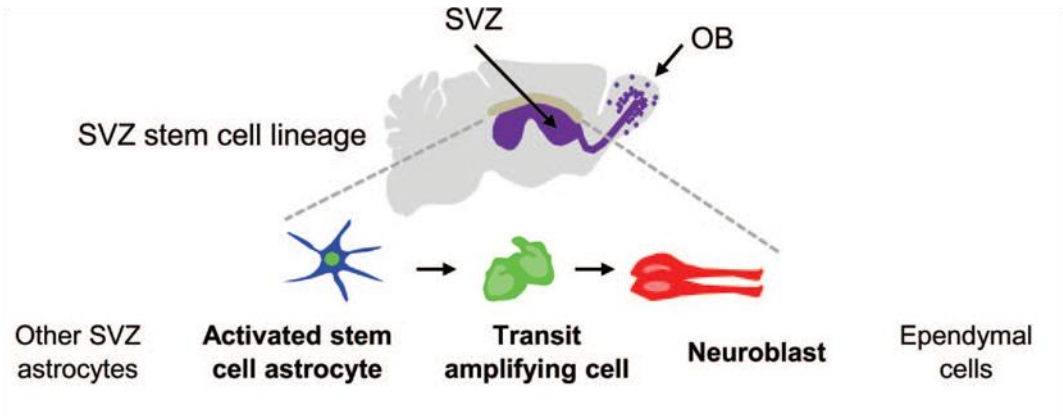


Figure 1.6: Schematic representation of neurogenesis in the adult rodent brain

The adult neurogenic niches, namely the SVZ and the dentate gyrus (DG) of the hippocampus hold the adult NSCs. While the DG provides neurons to the proximity, the SVZ provides the OB with new neurons via the RMS. Once the new cells reach the OB they differentiate further. While moving from the SVZ to the OB, the cells mature and the change from the activated stem cell astrocyte stage via the transit amplifying cell stage to a neuroblast (figure adapted from Pastrana et al., 2009).

The SGZ is a region within the hippocampus and it is the second key neurogenesis site and neurogenesis occurs mainly in locations close to blood vessels (Palmer et al., 2000). In contrast to SVZ neurogenesis, where neurons are born relatively distant from the location where they are needed, SGZ neurogenesis occurs in proximity to where the new neurons are needed. In the hippocampus, the newly formed neurons have a role in some forms of memory formation such as long term spatial memory (Snyder et al., 2005) as well as in cognitive flexibility (Burghardt et al., 2012). Spatial learning influences dendritic arbor formation of new neurons if they are functionally integrated into the hippocampus (Lemaire et al., 2012). The newly generated neurons are more excitable and more likely to produce a stable LTP (Saxe et al., 2006). This brain area has also been shown to be sensitive to damage at early stages of Alzheimer's disease and seem to be entangled with neurogenesis, potentially being involved in facilitating the progression of the disease, but may also be involved in recovery of functions (Mu and Gage, 2011).

1.2.2. Regulation of neurogenesis

Several aspects of the neurogenic niches contribute to the regulation of neurogenesis. Cell-cell interactions, as well as the contact to the cerebrospinal fluid or interactions with blood vessels, help form these complex regulatory mechanisms. Furthermore, the extracellular matrix, as well as the epigenetic state of the cells influences the NSCs (Lim and Alvarez-Buylla, 1999; Song et al., 2002).

Growth factors play a vital role in neurogenesis, and a mitogenic effect on SVZ stem cells has been reported, while others contribute to the differentiation mechanisms (Panchision and McKay, 2002). Two of these factors are EGF (epidermal growth factor) and FGF-2, which have been shown to have mitogenic activity through different mechanisms in the SVZ and furthermore might be involved in cell fate decisions (Lennington et al., 2003; Maric et al., 2007). The FGFR, as well as the EGFR (epidermal growth factor receptor), are expressed in the neurogenic niche, and SVZ astrocytes express FGFR, whereas EGFR expression was observed in B-cells and C-cells (Morshead et al., 1994; Doetsch et al., 2002). In FGF-2 KO animals, a 50% reduction of SVZ neurogenesis was observed with a knock on effect on the number of new neurons in the OB. As a result, the OB of these animals is smaller when compared to wild type (wt) animals. A loss of stem cells, rather than impaired proliferation causes the reduced stem cell numbers (Zheng et al., 2004). *In vitro* NSCs require growth factors like EGF or FGF-2 to expand. They can be either grown as neurospheres or as adherent cell cultures (Temple, 2001; Conti et al., 2005). It should be mentioned that EGF-responsive neurospheres typically arise from transit-amplifying C-cells and not *in vivo* stem cells (Doetsch et al., 2002). Therefore, C-cells have stem cell potential *in vitro*, which is further regulated *in vivo*. Another set of experiments supports the difference between the two signaling mechanisms. When EGF or the endogenous EGFR ligand tumor growth factor (TGF)-α were given via intracerebroventricular infusion an induction of mitotically active, functional, radial-glia like cells from progenitor cells was observed in the adult SVZ. This was not observed for FGF-2 (Gregg and Weiss, 2003).

There is a variety of vascular factors affecting the neurogenic niche in the SVZ as well as the SGZ. Endothelial cells are located around the lumen of the blood vessels, and the basal lamina separated them from the rest of the brain. Neurogenesis often occurs in the proximity of blood vessels (Palmer et al., 2000). It has been reported that co-culture with endothelia cells inhibits NSC cell differentiation (Shen et al., 2004). They secrete many different factors known to be mitogenic or to be involved in differentiation or survival such as FGF-2, IGF-1 (insulin like growth factor 1), vascular endothelial growth factor (VEGF), Platelet derived growth factor (PDGF), interleukin 8, and BDNF. Moreover, they are influenced by FGF-2, IGF-1 and TGF-α (reviewed in Doetsch, 2003b). Noggin, which antagonises bone morphogenic protein (BMP) signaling, is produced by ependymal cells and inhibits BMP dependent glial differentiation (Lim et al., 2000). Together this illustrates the significant role of vascular factors in neurogenesis.

Furthermore, neurotransmitter signaling has been linked to adult stem cell niche regulation and is discussed elsewhere (Hoglinger et al., 2004; Vaidya et al., 2007; Conover and Notti, 2008). Many regulatory mechanisms are similar between tumor stem cells and NSCs and EGF, FGF, Sonic hedgehog homolog (shh) and Notch signaling has been demonstrated to be significant for tumour maintenance and initiation (Sanai et al., 2005). This is an additional aspect, which makes it essential to understand the regulation of NSCs, as it might be transferrable to cancer stem cell research and tumour treatment.

1.2.3. Changes in neurogenesis in disease

Several neurodegenerative diseases have been linked to changes in neurogenesis. A reduced amount of proliferation is observed in the SVZ leading to a compromised olfaction, which is used as an early diagnostic indication for human Parkinson's disease cases. In these patients, the dopaminergic input into the SVZ, which is known to have a regulatory role for the stem cell niche is reduced, leading to a reduced SVZ neurogenesis. Therefore, rather than being the cause of Parkinson's disease it is a consequence. However, early treatment of Parkinson's disease incorporates an increased exercise regime thought to be beneficial via multiple mechanisms including increased neurogenesis (Alonso-Frech et al., 2011).

A misregulation of neurogenesis is also observed in Alzheimer's disease. While increased proliferation coupled to an increased expression of immature neuronal markers has been reported in the hippocampus (Jin et al., 2004) others have shown a decrease in SVZ neurogenesis in Alzheimer's disease patients (Curtis et al., 2007). Overall proliferation in the neurogenic niches seems reduced within the CNS in Alzheimer's disease animal models (reviewed in Abdipranoto et al., 2008). Furthermore, amyloid β plaques, which are closely linked to Alzheimer's disease, inhibit human NSC proliferation and differentiation *in vitro* (Abdipranoto et al., 2008). A discussion on a neurogenesis based strategy for therapy or diagnosis can be found elsewhere (Mu and Gage, 2011).

An impaired neurogenesis process has also been shown to have an effect on mood and depression. Studies have shown a connection between low levels of neurogenesis and depression (Sapolsky, 2004), while others showed that impaired neurogenesis does not automatically lead to depression (Vollmayr et al., 2003). A variety of antidepressants increase neurogenesis in the DG and neurogenesis furthermore has been shown to be required for the effect of antidepressants (Santarelli et al., 2003). Others doubt the logic applied in this publication based on the used methodology (Phillips et al., 2006). Therefore, even though changes in neurogenesis might not be the direct cause of depression, they might underlie the long-term tendency as well as nature for depression to reoccur (Phillips et al., 2006). A recent study investigated the effect of antidepressants on human with a serious depressive disorder. They found that hippocampal neural progenitor cells and angiogenesis are selectively increased in the mid DG as well as the anterior. As has been seen in other tissues where proliferation occurs, these results point potentially towards a relationship between neurogenesis and angiogenesis and furthermore might offer a potential insight into the role of theses mechanisms for the actions of anti-depressive drugs (Boldrini et al., 2012).

1.2.4. Adult neurogenesis in humans

As for most biological mechanisms the majority of research on adult neurogenesis is performed on animal models such as rodents in combination with *in vitro* research. There is a vast variety of promising results regarding neurogenesis, RMS migration, and OB neurons based on rodent research. The leading question is: "How similar is adult human neurogenesis to that seen in rodents?"

A study based on 5 human cancer patients provided the first solid evidence for adult neurogenesis in humans. The patient had been given BrdU as part of their tumour diagnostic treatment. Post mortem tissue from these cases showed BrdU labelled cells in the SGZ as well as in the SVZ (Eriksson et al., 1998). The extent and relevance of human neurogenesis is discussed until today. Optimistic views argue in favour of a functional, anatomically altered RMS (Curtis et al., 2007; Wang et al., 2011) and progenitors within the human OB itself (Pagano et al., 2000). Interestingly, another migratory stream has been identified in humans targeting the prefrontal cortex. This might fill the need for an increased regional complexity in humans compared to animals (Sanai et al., 2011). A particularly clever way to investigate human neurogenesis was used by Bergmann and colleagues based on ^{14}C released by atom testing in 1963. Since then ^{14}C levels have steadily declined allowing to judge the "age" of a neuron based on its ^{14}C concentration it integrated at its birth. If a neuron has a different "age" from the surrounding cells, this indicates, that neurogenesis must have taken place. Only a limited number of new cells were found in the human OB in this study (Bergmann et al., 2012). While the technique is very promising and holds a lot of potential, the choice of patients, the relevance of the OB and a good sense of smell in the studied patients is discussed critically by the authors (Bergmann et al., 2012; Macklis, 2012). Results in mice also saw considerable death of adult born neurons if the mice were not exposed to new smells (Lazarini and Lledo, 2011). This raises the question to whether adult neurogenesis in humans might be missed or underestimated due to the failure of new neurons to survive. It would be interesting to use the ^{14}C method to investigate other neurogenic brain regions in humans as has been done by Eriksson and colleagues (Eriksson et al., 1998). Therefore, even though the extent of human

neurogenesis is still debated, the majority of data point towards at least small amounts of neurogenesis in the adult human brain.

1.2.5. The role of the eCB system in neurogenesis

The different components of the eCB system are expressed by neural progenitor cells and have been suggested to play a prominent role for their proliferation. DAGLα is expressed by the ependymal cells lining the lateral ventricle and by cells displaying proliferation markers (Goncalves et al., 2008). It was also found to be present in cultured neurospheres and different NSC lines (as well as DAGLβ) where it is essential for their proliferation (Molina-Holgado et al., 2007; Goncalves et al., 2008). The DAGL inhibitor RHC80267 can suppress the proliferation of cells in the SVZ in young mice. Together these results indicate that the DAGLs are needed for NSC proliferation. Furthermore, RHC as well as THL significantly reduce the number of new neurons in the OB, which is due to a lack of neuroblasts migrating through the RMS (Goncalves et al., 2008). Neurogenesis displays dependence on different DAGL isoforms in the different brain regions. In DAGLα KO mice, a 50% reduction in proliferating cells was observed in the SVZ in young adult mice, with a similar reduction seen in the DG of the hippocampus. Knocking out DAGLβ however only leads to a reduced proliferation in the DG (Gao et al., 2010). Taken together, these results show the importance of DAGL for adult neurogenesis.

Cells expressing the CB1/2 receptors typically also express DAGL suggesting an autocrine signaling mechanism. It has been demonstrated, that 2-AG can increase the number of BrdU positive NSCs, and this occurs in a CB1 receptor dependent manner (Aguado et al., 2005). CB1 and CB2 receptors are expressed by NSC as well as in stem cell niches (Aguado et al., 2005; Aguado et al., 2006; Palazuelos et al., 2006; Goncalves et al., 2008) and are also needed for the proliferation of cultured NSCs (Goncalves et al., 2008). More recently the CB2 receptor has been shown to promote neural progenitor proliferation through the mTOR signaling cascade (Palazuelos et al., 2011). Progenitor proliferation and astrogliogenesis are impaired in CB1 receptor KO mice further emphasizing the role of the eCB system in neurogenesis. The 2-AG degrading enzyme FAAH is expressed by proliferating adult neural progenitor cells and postnatal glial cells as well (Aguado et al., 2005).

Increasing eCB levels by using FAAH inhibitors can also stimulate neural progenitor proliferation in CB1 receptor deficient mice (Aguado et al., 2006). A similar result has been seen using a neurosphere assay, where CB1 and/or CB2 receptor activation increases progenitor proliferation. This effect is mirrored by the use of FAAH inhibition, leading to an increase of eCBs (Aguado et al., 2005; Palazuelos et al., 2006; Molina-Holgado et al., 2007). Inhibition of CB1 or CB2 receptors ,on the other hand, decreases NSC proliferation, while it has no effect on their survival (Goncalves et al., 2008). This overall shows the role of CB1 and CB2 receptors in progenitor proliferation.

Surprisingly, CB2 receptor antagonism, but not CB1 receptor antagonism is associated with a reduction in neurogenesis in the SVZ of young adult mice. This has also been seen for the DAGL inhibitor, and a substantial reduction in neuroblast numbers migrating through the RMS was observed. To test if agonism can increase neurogenesis, a CB1/2 receptor agonist, or CB2 receptor agonists were used to treat young animals, but only a very modest increase of neurogenesis was observed. This might reflect that at a young age, eCB dependent neurogenesis might be occurring at a near optimal level and cannot be increased much further. However, in older animals where neurogenesis is dramatically reduced, it was possible to increase neurogenesis substantially with CB2 receptor agonists or FAAH inhibitors (Goncalves et al., 2008), suggesting that the age-related decline might reflect a run-down in eCB signaling.

1.2.5.1. The role of the eCB system in neuronal migration and integration

After the generation of new neuroblasts in the lateral wall of the ventricle, these cells have to migrate to the correct location and differentiate into mature neurons. Recently, the eCB system has also been shown to play a prominent role in neuroblast migration in the RMS of young adult animals (Oudin et al., 2011a). Previously, their role in other migratory events has been shown in the context of tumour cell migration (Hermanson and Marnett, 2011). The newly found role in neuroblasts migration is especially intriguing, since these cells have been reported to migrate to regions of injury and therefore could be important for brain repair.

Mouse migratory neuroblasts express DAGL as well as MAGL. In a cell based migration assay, DAGL inhibition, as well as CB1/2 receptor inhibition decreased migration. Additionally the eCB system *in vivo* has a role in morphology changes, which are associated with migration like nucleokinesis or process branching (Oudin et al., 2011b). This indicates that the DAGL-eCB system is involved in neuroblast migration. As mentioned above, CB2 receptor agonist treatment not only increased SVZ proliferation, but also increased the generation of new neurons in the OB, two weeks after treatment (Goncalves et al., 2008). Therefore, eCBs also have a role in the integration of new neurons.

1.2.5.2. eCB requirement for neurogenesis changes with age

Neurogenesis in the SVZ drastically decreases between month 2 to 22 in mice (Luo et al., 2006) and a similar reduction in neurogenesis with age has been observed with humans (Knoth et al., 2010). Comparing 6 week old mice with 6 month old mice, the neurogenesis levels in the SVZ dropped by ~80% and was almost not existent by 20 month (Goncalves et al., 2008). 6 month old mice showed increased cell proliferation by approximately 3.5 fold in the SVZ after CB2 receptor agonist treatment, but not CB1 receptor agonist treatment. This effect can be prevented by the use of a CB2 receptor antagonist. The increased proliferation was linked to an increased number of new neurons in the OB 2 weeks later (Goncalves et al., 2008). The CB1/CB2 receptor agonists Win-55,212-2 displayed a similar effect to the CB2 receptor agonist, as it increased proliferation by 3.5-fold and was prevented by a CB2 receptor antagonist. FAAH KO can induce hippocampal progenitor proliferation (Aguado et al., 2005) and blocking eCB degradation by inhibiting FAAH with URB597 resulted in a similar CB2 receptor dependent increase in proliferation (Goncalves et al., 2008). To test if the effect was even bigger in older mice, 20 month old mice were tested and display a 6-fold proliferation increase. This retuned them to the neurogenesis levels of a 6-month old mouse (Goncalves et al., 2008). These experiments display the eCB dependency of neurogenesis especially in old age and furthermore environmental factors such as high fat diet have been demonstrated to influence eCB dependent neurogenesis. The CB1 receptor antagonist AM251 can alter the proliferation in mice, which are

fed a high fat diet. This has different effects on different brain regions. In the SVZ a decrease of BrdU positive cells was found, while an increase was identified in the SGZ (Rivera et al., 2011). Therefore, the effect of eCB signaling on neurogenesis might be limited to some life conditions and stages.

1.3. The therapeutic potential of the eCB system

The eCB system has many roles within the CNS and beyond and its misregulation is involved in a range of diseases. Moreover, certain symptoms such as pain can be addressed via modulation of eCB signaling. Therefore, it is not surprising that the eCB system provides a promising target for therapeutic purposes and indeed several drugs are now in clinical trials. The many medicinal effects of *cannabis sativa* on the human body and mind have been known since it was described 2700 BC, but it took until the 20th century to start understanding the underlying mechanism. The increased understanding of the relationship between the eCB system and diseases allows us to design new treatment strategies as well as develop new drugs.

Here, we will focus on the role of the eCB system in obesity and neuroprotection, but further connections between the eCB system and diseases have been made in the area of anxiety and depression, pain and inflammation as well as liver disease and osteoporosis (Di Marzo, 2008; Fowler et al., 2010). In many cases, it has been shown that the eCB system can have enhancing as well as reducing effects on symptoms, which displays the complexity of the system.

1.3.1. Eating disorders and obesity

Considering the well established appetite increasing effect of cannabis it is not surprising that eating disorders are on the list of diseases to be potentially addressed by targeting the eCB system. Alterations in eCB levels might be an adaptive response to encourage eating or to deal with the lack of food. It furthermore was linked to a change in appetite causing overeating which in turn results in fat accumulation and/or obesity (reviewed in Di Marzo 2008). The eCBs regulate appetite though their pro-orexiogenic mechanisms in the hypothalamus. Adipocytes release leptin which leads to a reduction of 2-AG as well as anandamide levels (Di Marzo, 2008).

Some results point to adult neurogenesis occurring in the hypothalamus and moreover it might causally influence the regulation of energy balance (Kokoeva et al., 2005). This is interesting because a connection between diet induced obesity and CB1 receptor dependent regulation of proliferation has been recently reported. This observation provides support for the notion that neurogenesis is regulated via CB1 receptor signaling as a response to obesity (Rivera et al., 2011). Mice were fed a low or high fat diet and treated with AM251. AM251 changed food intake and weight in either case but interestingly only in the animals receiving a high fat diet showed an effect on proliferation. This might indicate that the observed proliferation is not dependent on caloric intake but on the type of nutrient (Cota and Marsicano, 2011; Rivera et al., 2011).

A change in hippocampal eCB signaling also has been observed in diet-induced obese mice. eCB-mediated synaptic plasticity was especially affected within the CA1 region. Here, an enhancement of long-term depression of inhibitory synapses and DSI was observed (Westbrook et al., 2008). Food availability can change the output of the hypothalamic nuclei, which underlies energy homeostasis. GABA synapses in the dorsomedial hypothalamus, a part of the brain known to be involved in satiety signals, display eCB dependant LTD and were the focus of this study. 24h of food deprivation causes a loss of CB1 receptor signaling resulting in the synapses only being capable of LTP, but not LTD. Therefore, the regulation of CB1 receptor signaling can act as a switch between LTD and LTP at GABAergic synapses (Crosby et al., 2011).

In summary, the eCB system was shown to react as well as act upon energy level changes and is an excellent target to address eating disorders. It has been shown that CB1 receptor agonist treatment in low doses increases food uptake, while CB1 receptor antagonist decreases food uptake as well as body weight (Mackie, 2006). This has been exploited by developing CB1 receptor antagonists as novel drugs against obesity such as rimonabant and taranabant (Di Marzo, 2008).

1.3.2. Neuroprotective effects of eCB signaling

The eCB system is involved in providing neuroprotection following neuronal damage. This damage can be either acute, like trauma or ischemia, or chronic as caused ,for example, by Alzheimer's disease (Fowler et al., 2010). It has been demonstrated that 2-AG levels are significantly increased following close head injury in mice. Mice treated with synthetic 2-AG after head injury displayed reduced brain oedema, an improved clinical recovery, a decreased infarct volume as well as decreased hippocampal cell death. The positive effect of 2-AG could be reduced in a dose dependent manner with the use of CB1 receptor antagonist SR-141761A (Panikashvili et al., 2001). A neuroprotective effect was also observed when CB1 receptor agonists were used in a multiple sclerosis model (Croxford et al., 2008). CB1 receptor KO mice helped to support the notion of the protective effects of CBs, as CB1 receptor KO mice showed more severe symptoms after a stroke. They died more frequently from focal cerebral ischemia, and the infarct size was increased amongst other symptoms. This indicates that the CB1 receptor dependent eCB pathway protects the mouse brain against at least some aspects of stroke (Parmentier-Batteur et al., 2002). Furthermore, CB1 receptor KO mice recovered more slowly from close head injury compared to wt mice (Panikashvili et al., 2005). These and other results demonstrate that the eCB can have a neuroprotective effect and together with other aspects of eCB signaling, have raised hope in the context of different neurodegenerative diseases such as Alzheimer's disease or multiple sclerosis (Fowler et al., 2010; Karl et al., 2012; Pryce and Baker, 2012). However, the inverse CB1 receptor agonist rimonabant also has been demonstrated to have neuroprotective properties in some animal models (Fowler et al., 2010), which indicates that in some context eCB mediated mechanisms can have a neurotoxic effect as well. Therefore, further research is needed to determine the subtle details of which aspects of eCB signaling are neuroprotective.

1.4. Aims and objectives

At the start of my project, our lab was strongly involved in researching the role of eCB signaling in adult neurogenesis in the SVZ as well as NSC proliferation (Goncalves et al., 2008). This went hand in hand with an interest in the involvement of eCB signaling in RMS migration (Oudin et al., 2011b). Since cloning the DAGLs in 2003 (Bisogno et al., 2003) our lab was particularly interested in DAGL depended eCB signaling. Very little information is available on what is driving the eCB system in NSCs and possible mechanisms for DAGLα regulation (discussed in Reisenberg et al., 2012).

As the brain is a highly heterogeneous tissue, it is extremely challenging to uncover molecular mechanisms *in vivo*. The Cor-1 cells are a NSC model (Conti et al., 2005) and were used as an *in vitro* model for our proliferation and migration studies. Cell proliferation, migration, and differentiation are the key processes occurring during neurogenesis as well as during neural development and relevance for the eCB system was identified for the first two steps. Moreover, the Cor-1 cells express the core components of the eCB signaling machinery and require basal eCB tone for proliferation and migration (Goncalves et al., 2008; Oudin et al., 2011b). Therefore, they are a suitable model system to investigate eCB signaling in NSCs.

We wanted to address the following questions:

- Does eCB signaling regulate the differentiation of Cor-1 cells to neurons or glia?
- What drives eCB signaling in NSCs and what are the downstream effectors?
- Do DAGLα levels change during the differentiation of Cor-1 cells into neurons or glia?
- Can we establish an assay to analyse DAGL activity?
- Is DAGL regulated by post translational modified such as ubiquitination or phosphorylation?

CHAPTER II: Material and Methods

2.1. Materials

All reagents were purchased by Sigma-Aldrich or VWR unless otherwise stated.

Cor-1 cell lines

Cor-1 cells are ES cell derived mouse NSCs and were a kind gift from Austin Smith (Conti et al., 2005).

Cor-1 cells were transfected with plasmids (pCDNA3.1D/V5-His-TOPO) containing the human DAGLα or mouse DAGLβ gene (plasmids Praveen Paul and Fiona Howell) and stable cell lines were established and named V5α7 and V5β5 respectively (Philipp Sütterlin).

A stable Cor-1 cell line expressing the human DAGLα in pcDNA3.1D/V5-His-TOPO vector with mutations in the putative destruction (d-) box motive were established for this study. The amino acid sequence was changed from ^{892}R-G-E-L-A-L-H-N-G to ^{892}A-G-E-A-A-L-H-N-G and the mutations of the putative d-box were carried out and confirmed by Exonbio. A stable cell line was established expressing the d-box mutated DAGLα-V5 construct and named Mα8.

Phosphate buffered saline (PBS)

One tabled (Oxoid) containing 0.20g/l KCl, 0.20g/l KH_2PO_4, 8g/l NaCl, 1.15g/l Na_2HPO_4 dissolved in 100ml distilled water

LB media and LB agar

25g Luria bertani (LB) broth powder (Merck) or 37g LB agar powder (Merck) was added to 1l of water and autoclaved. 50μg/ml ampicillin was added if required.

PBST

PBS plus 0.1% Tween20

Tris buffered saline (TBS)

3.029g Tris (hydroxymethyl)-methylamine (BDH) and 4.383g NaCl (BDH) dissolved in 500ml distilled water and adjusted to pH 7.4

TBST

TBS and 0.1% Tween20

Lysis buffer (ODG for Cor-1 cells)

50mM TRIS pH8, 150mM NaCl, 10mM $MgCl_2$, 1% triton-X, 5% glycerol, 10mM NaF, 1mM sodium orthovanadate, 1mM PMSF and complete protease inhibitors (Roche).

Lysis buffer (brain tissue)

50mM Tris pH7.5, 150mM NaCl, 1% triton-X, 10mM NaF, 1mM sodium orthovanadate, 1mM PMSF and complete protease inhibitors (Roche).

Lysis buffer for membrane extraction (DAGL activity assay)

20mM HEPES (pH 7), 2mM DTT, 25M Sucrose, protease inhibitor, 10mM NaF, 10nM NaVO3

Membrane buffer (DAGL activity assay)

20mM HEPES (pH 7), 2mM DTT, protease inhibitor, 10mM NaF, 10nM NaVO3

5x Protein loading buffer

300mM Tris pH6.8, 25% glycerol, 10% SDS, 0.015 bromophenol blue, 500mM DTT

Table 2.1: SDS-polyacrylamide gel

	7.5% running gel	*5% stacking gel*
30% acryl amide/Bis (VWR international)	2.5ml	1.67ml
1.5M Tris, pH 8.8 (VWR international)	3.7ml	1.25ml
10% SDS (VWR international)	100ul	100ul
H_2O	3.63ml	6.91 ml
TEMED (National diagnostics)	6.6ul	20ul
10% Ammonium persulphate (VWR international)	66ul	50ul

Table 2.2 Buffers for western blot analysis

	10x running buffer	10x transfer buffer
Tris Base	30.3 g	30.3 g
Glycine	144.2 g	144.2 g
SDS	10 g	-
Methanol	-	200 ml
made up to 1 L with distilled H_2O		

Fixative for immunocytochemistry

PBS with 4% par formaldehyde (PFA), 10% sucrose, pH7.4

Blocking and permeabilising buffer for immunocytochemistry

PBS with 0.1% Troton-X-100, 1%BSA and 0.1% Na azide

Table 2.3: Primary antibodies for western blotting

Antibody	*Dilution*	*Species*	*Company*
Anti-V5	1:1000 - 1:5000	Mouse	Invitrogen
GFAP	1:20000	Rabbit	Dako
Tuj-1	1:500	Mouse	Abcam
Tuj-1	1:500	Rabbit	Covance
Anti-V5	1:3.000	Rabbit	Invitrogen
Nestin	1:20	Mouse	DSHB
DAGLα	1:1000	Rabbit	Gift from Watanabe
DAGLα	1:1000	Guinea pig	Gift from Watanabe
DAGLβ	1:500	Rabbit	Eurogentech
CB1 receptor	1:1000	Rabbit	Gift from K.Mackie
Ubiquitin	1:250	Mouse	Stressgen
Ubiquitin	1:2000	Rabbit	Abcam
p70 S6 kinase	1:1000	Rabbit	Cell signaling
Phospho P70 S6 kinase	1:1000	Rabbit	Cell signaling

Sox2	1:1500	Rabbit	Chemicon
Actin	1:5000	Mouse	Abcam
InsR β	1:100	Mouse	Abcam
FAAH	1:1000	Mouse	Abcam

*affinity purified against the SSDSPLDSPTKYPLT epitope of DAGLβ

Table 2.4: Secondary Antibodies for western Blotting

Antibody	*Dilution*	*Species*	*Company*
Anti-mouse HRP	1:3000	Horse	Vector Laboratories
Anti-goat HRP	1:3000	Horse	Vector Laboratories
Anti-rabbit HRP	1:3000	Horse	Vector Laboratories
Anti-mouse Alexa Fluor 700	1:3000	Goat	Invitrogen
Anti-rabbit Alexa Fluor 800	1:3000	Goat	Invitrogen

Table 2.5: Primary Antibodies for immunofluorescence

Antibody/Dye	*Dilution*	*Species*	*Company*
Anti-V5	1:500	Mouse	Invitrogen
GFAP	1:2000	Rabbit	Dako
Tuj-1	1:500	Mouse	Abcam
Tuj-1	1:500	Rabbit	Covance
Anti-V5	1:3.000	Rabbit	Invitrogen
Nestin	1:20	Mouse	DSHB

DAGLα	1:500	Rabbit	Gift from Watanabe
DAGLα	1:500	Guinea pig	Gift from Watanabe
CB1 receptor	1:100	Rabbit	Gift from K.Mackie
InsR	1:200	Rabbit	Santa Cruz
InsRβ	1:50	Mouse	Abcam
IGF-1	1:100	Rabbit	Abcam
Phalloidin AlexaFluor 488	1:100		Molecular probes
Hoechst 33342(blue)	1:10.000		Invitrogen

Table 2.6: Secondary Antibodies for immunofluorescence

Antibody	*Dilution*	*Species*	*Company*
Anti-mouse IgG Alexa594	1:1000	Goat	Molecular probes
Anti-rabbit IgG Alexa594	1:1000	Goat	Molecular probes
Anti-guinea pig Alexa594	1:1000	Goat	Molecular probes
Anti-mouse IgG Alexa488	1:1000	Goat	Molecular probes
Anti-rabbit IgG Alexa488	1:1000	Goat	Molecular probes

Table 2.7: Drugs used on Cor-1 cells

Drug	*Target*	*Concentrations used*	*Company*
ACEA	CB1 receptor agonist	0-1µM	Tocris
AG1024	IGFR and InsR tyrosine activity inhibitor	0-100nM	Calbiochem (Merck)
AG1478	EGFR inhibitor	500nM	Biomol
AM251	CB1 receptor antagonist	0-1µM	Tocris
AM630	CB2 antagonist	0-1µM	Tocris
Go6976	PKC inhibitor	0-1µM	Tocris
H-1356	IGFR inhibitor	0-1µM	Bachem
JTE-907	CB2 receptor antagonist	0-1µM	Tocris
JWH-133	CB2 receptor antagonist	0-1µM	Tocris
KN62	CaM kinase inhibitor	0-1µM	Tocris
Lacatcystin	Proteasome inhibitor	0-0.5µM	Sigma
LY320135	CB1 antagonist	0-1µM	Tocris
OMDM-188	DAGL inhibitor	0-1µM	Kind gift from the DiMarzo lab
PD173074	FGFR inhibitor	500nM	Calbiochem (Merck)
PD168393	EGFR inhibitor	0-500 nM	Calbiochem (Merck)
Rapamycin	mTOR inhibitor	0-2µM	Sigma
Roscovitine	CDK1,2,5 kinase	0-10µM	Cell signaling

Mowiol mounting solution

PBS with 17% mowiol 4-88 (Calbiochem) and 33% glycerine

Trypsin

1mM EDTA filter sterilised, 1% chick serum, 0.025% trypsin (Invitrogen)

Table 2.8: In-house N2

In-house preparation	Final conc. in media	Supplier	Solvent
Insulin	25µg/ml		0.01M HCl
Apo-transferrin	100µg/ml		Water
BSA	50µg/ml	Gibco	
Progesterone	20ng/ml		EtOH
Putrascine	16µg/ml		Water
Selenium Chloride	5.18ng/ml		Water

2.2. Methods

2.2.1. Cell culture

Passaging

Cor-1 cells were grown at 8% CO_2 and 37°C in growth media in T75 tissue culture flasks or 10cm dishes (Nunc) coated with 0.1% gelatine in PBS for 15 min to prevent neurosphere formation. For passaging, Cor-1 cells were trypsinised for <1 min with 1ml trypsin, washed off and collected in 9ml growth media, spun down at 240 g for 3min, before finally being resuspended in fresh growth media.

Cells used were from passage 25-40 and only used for experiments when morphology and proliferation rate appeared normal.

Cor-1 growth media

NS-A media (Cadama) supplemented with N2 (Invitrogen) or in house N2, L-glutamine, 10ng/ml EGF and 10ng/ml FGF2 (both from Peprotech).

Cor-1 seeding for immunocytochemistry

For the immunocytochemistry experiments, 13mm glass cover slips in individual wells of 4-well culture dishes (Nunc) were coated with 500µl 0.1% gelatine solution and left for at least 15min at RT. The gelatine was removed before adding Cor-1 cells in growth medium typically at concentrations between 5,000-20,000 cells/well. Cells were incubated at 37^{o}C, in an 8% CO_2 incubator until they reach the desired level of confluence.

Cor-1 seeding for 96-well experiments

96-well plates (Nunc) were coated with 100µl 0.1% gelatine solution and left for 15min at RT. The gelatine was removed and Cor-1 cells were seeded between 3,000-10,000 cells/well and grown at 37°C in 8% $C0_2$. Drugs were typically added the next day in a small volume of media.

Freezing of Cor-1 cells

Cells were frozen down in 500µl growth media and 10%DMSO (dimethyl sulfoxide) in 1.8ml cryotubes (Nunc) as cell pellets. Cells were recovered by being grown in preheated growth media in gelatine coated 10cm dishes overnight. The next day, the media was replaced by fresh media to remove DMSO residuals.

2.2.2. Differentiation of Cor-1 cells

2.2.2.1. Neuronal differentiation

Plating of Cor-1 cells for experiments

Glass cover slips in 4-well culture plates (immunocytochemistry), 96-well plates, or 6-well dishes (western blotting) were coated with a 50µg/ml solution of Poly-L-ornithine (Sigma) and incubated overnight at 4°C. The next day the plates or dishes were washed three times with sterile water, and a 6µg/ml solution of laminin (Sigma) was added and incubated for at least 2h at 37°C in a humidified incubator. The cover slips/plates were washed three times with PBS before being use.

Neuronal cell culture

Cells were seeded at a concentration between 5,000 to 20,000 cells/well (coverslips) or 3,000 to 10,000 cells/well (96-well plates) or 100,000-500,000 (6-well plate) depending on the experiment in growth media and incubated overnight. The next day media was changed to the neuronal differentiation media.

Neuronal differentiation media

For neuronal differentiation NS-A media was supplemented with 2mM l-glutamine, N2, 5ng/ml FGF-2 and B27 at the concentration recommended by the supplier (Invitrogen). This media is referred to as *neuronal differentiation media* in the remainder of this report. Half the media volume was typically replaced at 2 or 4d for immunocytochemistry or after 2d for western blot analysis samples.

2.2.2.2. Astrocyte differentiation

Plating of Cor-1 cells for experiments

Cover slips/ 96-well plates/6-well dishes were coated with 0.1% gelatine and cells were seeded at a concentration between 5,000 and 500,000 cells/well in growth media depending on the experiment and incubated overnight. The next day, the media was changed to astrocytic differentiation media and the cells were differentiated between 1-7 days depending on the experiment.

Astrocytic differentiation media

For astrocytic differentiation NS-A media was supplemented with 1ng/ml BMP-4 (R&D systems). This media is referred to as *astrocytic differentiation media* in the remainder of this report. Alternatively 5% fetal bovine serum was used to differentiate the cells to astrocytes.

2.2.3. Cell proliferation assay

Time-lapse microscopy (IncuCyte™ imaging)

Cor-1 cells were grown in 96-well plates (Nunc) coated with gelatine in growth media and left to attach over night. Drugs were diluted in growth media and added to the 96-well plates the following day, and pictures were taken of cells while they

were still growing under incubation conditions using IncuCyte™ imaging for 24-48h. The integrated confluence metric from Incucyte™ is used as a surrogate for cell number (For further information see: http://essenbioscience.com/IMAGES/proliferationappnote.pdf). The growth rate over time was established by comparing the area under the curve, which was calculated using an Excel macro.

2.2.4. Derivation of clonal, transgenic cell lines

Transformation

1-5µg DNA was added to 50µl chemically competent *Escherichia coli (E.Coli)* DH5α cells (Invitrogen) and incubated on ice for 30min. Cells were heat shocked for 20sec in a 37°C water bath and incubated on ice for 2min. 900µl LB media was then added and the cells shaken at 37ºC for 1h at 6g before being spread onto ampicillin agar plates.

The High Speed Purification Protocol from Qiagen was used following the manufacturer's protocol.

Nucleofection

Nucleofection is a transfection method, which allows the efficient and reproducible transfer DNA and RNA into cells. The method is electroporation based and uses a device (Nucleofector), which offers optimized electronical parameters. In combination with cell specific reagents it is possible to transfer DNA and RNA directly into the nucleus and cytoplasm. Thus, it offers an excellent alternative to viral vectors to transfect even non-dividing cells. The Nucleofection Technology was invented by the Amaxa AG.

6-well plates were prepared by coating with 0.1% gelatine in PBS for 15min, adding growth medium and preheating in a 37°C incubator. 2µg of plasmid DNA are required, and 1-5 million cells are needed per nucleofection. The Cor-1 cells were harvested and centrifuged as has been done for the cell passaging, all traces of medium were removed before cells were resuspended in nucleofection solution V (Amaxa biosystems) (100µl/nucleofection). 100µl nucleofection solution with

resuspended cells and the appropriate plasmid amount was added to a cuvette. Nucleofection was performed using a Nucleofector II machine (Amaxa biosystems) on programme T-030. To increase survival cells were transferred into medium containing 10% serum for 5min and added to the previously prepared medium in the 6-well-plate.

Selection with G418

After nucleofection cells were grown on 0.1% gelatine coated 6-well plates for 2d in growth media before the growth media was supplemented with 400µg/ml G418. Media was then exchanged daily to remove cell debris, and the antibiotic selection was maintained until individual colonies were visible by eye. About 10d after the nucleofection colonies were picked and transferred onto gelatine coated 24-well plates where they were grown until 70% confluent, which means that they are ready to be transferred to 6-well plates. Finally, the cells are transferred to T75 tissue culture flasks and further expanded.

Cell lines were maintained in media containing 300µg/ml G418 and screened for successful insertion of the mutated DAGLα-V5 construct by western blotting and immunocytochemistry.

2.2.5. Immunocytochemistry

The Cor-1 cells were grown on glass cover slips suitably coated for the experiment (see above for details).

Cell fixation

The cells were fixed by adding equal amounts of 4% PFA (Sigma) and 10% sucrose typically after 24-48h to the growth media. After 20min, the medium was replaced with 500µl of the same fixative (4% PFA, 10% sucrose) straight onto the cells for 20min. Cover slips were then washed three times for 5min in PBS.

Antibody/ dye

Cover slips were incubated with 350µl blocking and permeabilising buffer for 1h at room temperature (RT) in order to permeabilize the fixed cells and prevent non-

specific antibody binding. The primary antibodies were made up in the same solution (250µl/well) and added to the cells at 4°C overnight or 1h at RT. After three washes in PBS the secondary antibody solution and Hoechst, also made up in blocking buffer, was added for 1h at RT while cover slips were protected from light. Following a final three washes in PBS, the cover slips were mounted on slides using mowiol (Sigma) and stored in a dark box at 4°C.

Imaging

Unless otherwise stated, immunocytochemistry pictures were taken on an Axiovision Apotome microscope.

2.2.6. Immunoprecipitation

Cells were grown overnight in expansion media, or 3d in differentiation media, in 10cm dishes. Cells were lysed as described below, 500µg protein and 2µg anti-V5 antibody were rotated at 4°C overnight.

To prepare the beads, 10mg protein A sepharose (Sigma) was added in 200µl cold PBS per immunoprecipitation (IP) and rotated at 4°C for 5min. The protein A sepharose beads were centrifuged at 11,000g, wash with 500µl cold PBS three times, and resuspended in 500µl lysis buffer. The beads were added to the lysate and rotate at 4°C for 1h and afterwards centrifuged at 11,000g. The protein A sepharose beads with protein were washed three times with 500µl cold lysis buffer. 50µl 2 x protein loading buffer (dilute with water from 5 x buffer) was added to the beads and boiled for 5min and centrifuged for 1min. 20µl supernatant was loaded on a SDS-poly-acrylamide gel to separated proteins and later on transfer onto nitrocellulose membranes. Samples were typically loaded with a lysate and a no antibody, "negative IP," as controls.

2.2.7. Western blotting

Cell lysis

Cells were cultured in 6-well-plates or 10cm dishes until reaching 70–80% confluence, washed in ice-cold PBS and lysed in 50-500µl lysis buffer. The lysate was collected in 1.5ml eppendorf tubes and left to rotate for 1h at 4°C followed by

centrifugation at 11,000g for 10min at 4°C to separate out cell debris. The supernatant was collected and stored at -20°C.

Protein concentration was typically measured with Pierce Protein Assay Kit following the manufacturer's protocol to ensure even protein loading for western blotting and calculate the required protein amount for the IPs.

SDS-PAGE

15-60µl sample in 5x protein loading buffer were separated on SDS-polyacrylamide gels at 120V for approximately 1.5h in running buffer. Next the samples were transferred to nitrocellulose membranes (Amersham) (100V for 60min) in transfer buffer.

Western Blot

The membrane was blocked in TBS and 5% milk at RT for 1h, and the primary antibody solution was applied overnight at 4°C or for 1h at RT. Next the membrane was washed 3 times in TBST for 10min and then incubated with secondary antibody solution at RT for 1h. The secondary antibody was chosen according to development method and suitable for the used primary antibody. After another three 10min washes in TBST, the blot was developed using Amersham Enhanced Chemiluminescence (ECL) reagents and exposed to X-ray film for HRP labelled secondary antibodies. For Alexa Fluor 700 or 800 labelled secondary antibodies Odyssey infra red imaging were used. Blots were stripped using Re-Blot Plus (Chemicon International) if required according to the manufacturer's guidance.

Quantification

ECL/ECL plus visualized western blots were scanned in at 2400dpi using a PerfectionV700 scanner (Epson) and quantified using ImageJ analysis software. In general, samples were loaded with an equal protein loading where possible and the protein of interest was normalised to an actin control.

Western blots, which were visualised using Odyssey infrared imaging, were quantified using the Odyssey software.

2.2.8. A surrogate substrate DAGL activity assay

The following protocol is a modification of the protocols described by Pedicord *et al* 2011.

2.2.8.1. Membrane based DAGL activity assay

Membrane extraction

Cor-1 cells were grown up in 10cm dishes or in 6-well plates until ~70% confluent. The cells were washed with ice cold PBS and resuspended in 500µl lysis buffer for membrane assay and homogenized with an electric, handheld homogenizer (Polytron PT1200 E) (3 times 7sec with 10sec breaks). The cell lysates were centrifuged at 100,000g for 30min at 4°C using an ultra centrifuge. Afterwards, the membrane pellets were resuspended in 250µl membrane buffer and homogenized with an electric homogenizer (3 times 7sec with 10sec breaks). Where possible the whole protocol was carried out on ice.

Coomassie (Bradford) Protein assay (Thermo Scientific)

A Bradford protein assay was carried out with each sample being tested at 5 different dilutions (3x) to ensure that the protein concentration lay within the linear range of the BSA standard curve. For further details see the protocol provided by the manufacturer.

DAGL activity assay using the chromogenic substrate PNP

A 96-well assay using the surrogate substrate 4-nitrophenol butyrate (Sigma-Aldrich) (PNP) was established. 50mM HEPES pH 7.5 was used as an assay buffer. A minimum of 3 wells were used per condition, and the mean is presented. Due to solubility problems, PNP was pre-diluted in DMSO (5% final concentration) and used at 250µM unless otherwise stated. Membranes were typically used at 12.5µg/ml FAC, and THL was used as a background control at a concentration of 1µM to inhibit DAGL activity and added to the membranes 5-10min before the substrate was added to half of the plate. The reactions were measured kinetically using the SpectramaxPlus (Molecular Devices) set at 400nm every 12sec for 10min. Using the Soft Max Pro software the reaction rate was calculated based on the

linear regression and it reflects how much of the surrogate substrate has been metabolised.

DAGL activity assay using the fluorogenic substrate DiFMU

A 96-well-assay using the surrogate substrate 6, 8-difluoro-4-methylumbelliferyl-octanoate (DiFMU) (Invitrogen) (PNP) was established. 50mM MES pH6.5 was used as an assay buffer and a minimum of 3 wells were used per condition. For consistency reasons DiFMU was pre-diluted in DMSO (5% final concentration) and used at 10µM unless otherwise stated. Membranes were typically used at 12.5µg/ml FAC and THL was used as a control at a concentration of 1µM to inhibit DAGL activity and added to the membranes 5-10 min before the substrate was added to half of the plate. The reactions were measured kinetically using the Flexstation (Molecular Devices) set at an excitation wavelength of 360nm and an emission wavelength set to 450nm, and the reaction was measured every 10 seconds for 10min. Using the Soft Max Pro software the reaction rate was calculated based on the linear regression of the first 10 min and reflects how much of the surrogate substrate has been metabolised. Typically the mean of 3-4 wells is presented.

2.2.8.2. Cell based DAGL activity assay

DAGL activity assay using the chromogenic substrate PNP

40,000 Cor-1 cells were seeded into 96-well plates and left overnight to attach. The growth media was carefully removed immediately before the assay was taken out and replaced with the adequate assay buffer (50mM HEPES pH 7.5). A minimum of 4 wells were used per condition and the mean is presented. Due to solubility problems PNP was pre-diluted in DMSO (5% final concentration) and used at 250µM unless otherwise stated and THL was used as a control at a concentration of 25µM to inhibit DAGL activity unless otherwise stated and added to the cells 5-10 min before the substrate was added to half of the plate. The reactions were measured kinetically using the SpectramaxPlus (Molecular Devices) set at 400nm and the reaction was measured every 12 seconds for 10 min. Using the Soft Max Pro software the reaction rate was calculated based on the linear regression and

reflects how much of the surrogate substrate has been metabolised. Typically the mean of 4 wells is presented.

DAGL activity assay using the fluorogenic substrate DiFMU

40,000 Cor-1 cells were seeded into 96-well plates and left overnight to attach and the growth media was carefully removed immediately before the assay was performed and replaced with the adequate assay buffer (50mM MES pH 6.5). A minimum of 4 wells were used per condition and the mean is presented. For consistency reasons DiFMU was pre-diluted in DMSO (5% final concentration) and used at 10µM unless otherwise stated. The membranes were typically used at 12.5µg/ml FAC (final assay concentration) and THL was used as a control at a concentration of 25µM to inhibit DAGL activity unless otherwise stated and added to half of the cells 5-10 min before the substrate was added. The reactions were measured kinetically using the Flexstation set at an excitation wavelength of 360nm and an emission wavelength set to 450nm and measured every 10sec for 10min. Using the Soft Max Pro software the reaction rate was calculated based on the linear regression and reflects how much of the surrogate substrate has been metabolised. Typically the mean of 4 wells is presented.

2.2.9. Microarray analysis

Cor-1 cells were grown until 70% confluent in 10cm dishes for two days. Next, the drugs were added to the fresh media at the following dilutions: control (0.1% DMSO), AM251 (1µM), LY320135 (1µM), AM630 (1µM), JTE-907 (1µM), AG1478 (100nM), PD168393 (100nM), PD173074 (500nM) (see Table 2.7 for further information on the drugs). Structurally independent antagonists against the CB1 and CB2 receptors as well as the EGFR receptor were used to reduce false-positive results based on off-target effects. Cells then were lysed in RLT buffer (Qiagen), scraped off the plates under sterile conditions, and transferred to 1.5ml eppendorf tubes. The samples were snap frozen on dry ice and the drug treatments were repeated 3-4 times on consecutive days. Then the samples were shipped to Wyeth Research (now Pfizer) on dry ice, where the mRNA was extracted and used to perform microarrays based on the Affymetrix GeneChip Mouse Genome 430 2.0

array. For further information on how the microarrays were conducted and analysed see (Suetterlin et al., Submitted)

2.2.10. Statistical analysis

Two sided student t-test or anova (analysis of variance) in combination with Tukey post-hoc testing was used as indicated. Some data was normalised setting the control to 100%. This was done when data sets showed a lot of experimental variation amongst sets.

CHAPTER III: Results 1

Characterization of Cor-1 cells and the role of eCB signaling for their differentiation

3.1. Introduction

The human brain contains a trillion neurons and a quadrillion of synapses (Ho et al., 2011) and their connections define human perception, thoughts and emotions. The brain is an extremely heterogeneous tissue making it challenging to uncover the molecular mechanisms underlying adult neurogenesis. A number of cell culture model systems to investigate how new neurons are generated in the adult brain have been developed. For example a "neurosphere" assay containing clusters of floating cells consisting of neural progenitor cells and their differentiating progeny has been adopted for this purpose by many labs (e.g Reynolds and Weiss, 1992; Morshead et al., 1994). They have provided many insights into the factors that regulate neurogenesis *in vitro*; however their heterogeneous composition imposes limitations. Three different homogeneous cultures of NSCs were established by the Austin Smith laboratory and kindly provided to us. While CGR8 and NS5 cells are derived from embryonic stem cells and maintained at the NSC state, Cor-1 cells are derived from the E 16.5 mouse cortex (Conti et al., 2005). This thesis will focus on Cor-1 cells as a model system. The Cor-1 cells grow as an adherent NSC population and express a range of NSC markers and like their endogenous counterparts they can be differentiated into neurons, astrocytes, and oligodendrocytes (Conti et al., 2005; Glaser et al., 2007). Furthermore, they express the different components of the eCB system and depend on eCB signaling for proliferation. They have proven to be a valuable tool to investigate the eCB system and establish their role in proliferation and migration (Goncalves et al., 2008; Oudin et al., 2011b).

3.2. Characterisation of Cor-1 cells and evidence that eCB signaling is important for their proliferation

Immunocytochemistry was used to confirm the continuous expression of NSC markers by the Cor-1 cells used in the present study. The Cor-1 cells are a relatively homogenous cell population displaying a polarised morphology and expressing the NSC markers nestin and Sox2 amongst other markers (see Conti et al., 2005). Under normal growth conditions they do not express the astrocytic marker/ ependymal cell marker GFAP or the neural lineage marker neuron-specific class III β-tubulin (Tuj-1) (Figure 3.1). The specificity of the anti-DAGLα and anti-DAGLβ antibodies was confirmed using cerebellar tissue from DAGLα and DAGLβ KO mice in comparison to wt tissue confirming the specific detection of the respective enzymes (Figure 3.2 A). Furthermore the expression of eCB system components was established and the expression of CB1 receptor, FAAH and DAGLα were confirmed by immunocytochemistry and/or western blotting (Figure 3.2 B+C). The expression of DAGLβ by Cor-1 cells has been shown elsewhere (Goncalves et al., 2008).

An *in vitro* proliferation assay has been established for Cor-1 cells. The total cell number for cultures up to 72h has been demonstrated to be highly correlated with their confluence, provided confluence remains below ~80% (Dr. Philipp Sütterlin; PhD thesis). Therefore, the proliferation of Cor-1 cells can be followed by measuring their confluence over time in culture. Cor-1 cells were cultured in 96-well plates at 8,000 cells/well and left to attach overnight. An automated image analysis platform that is based on capturing and analysis phase-contrast images (IncuCyte imaging technology and software) was used to measure confluence every 2h under normal cell growth conditions ± drug treatment.

Cor-1 cell proliferation can be inhibited by CB1 and CB2 receptor antagonists and by two DAGL inhibitors, namely RHC80267 and THL (Goncalves et al., 2008). A more selective DAGL inhibitor called OMDM-188 has recently been developed (Ortar et al., 2008) and the effect of this inhibitor on Cor-1 cell proliferation was determined for the first time in this study.

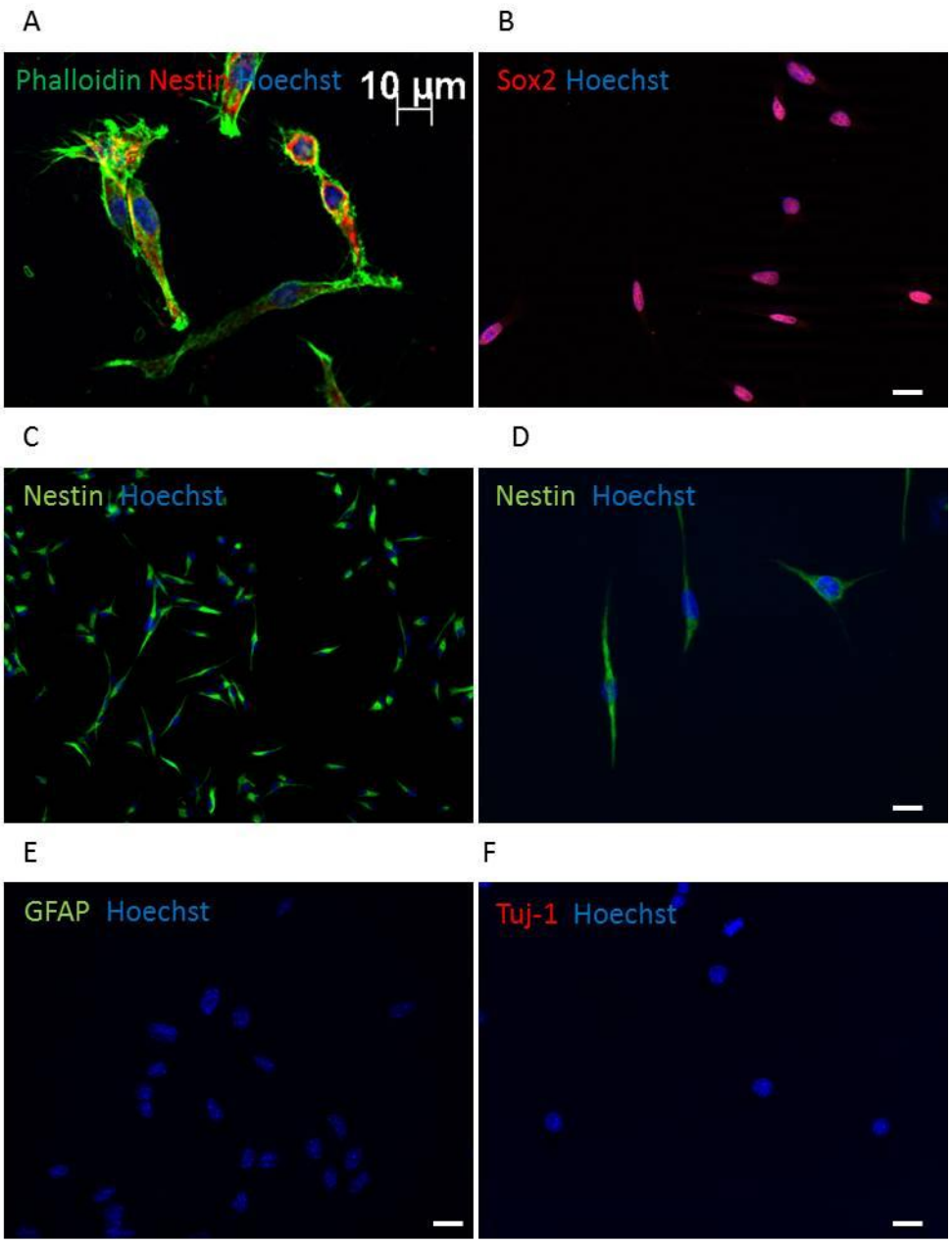
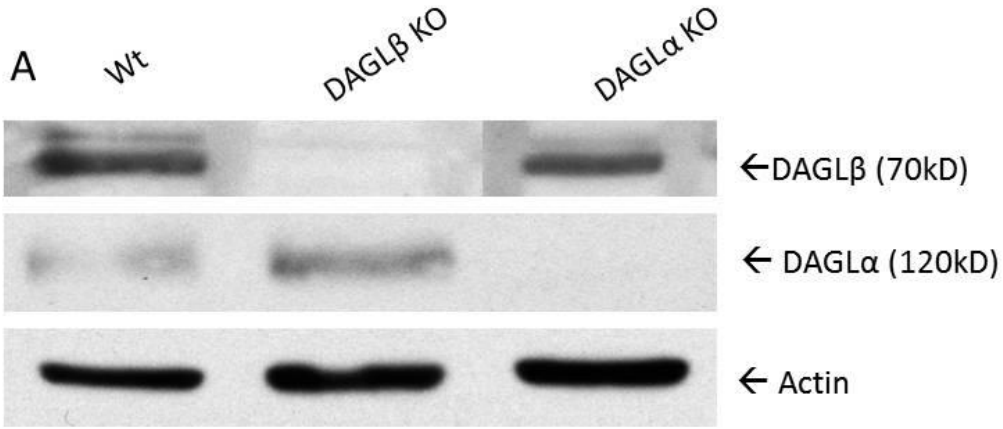
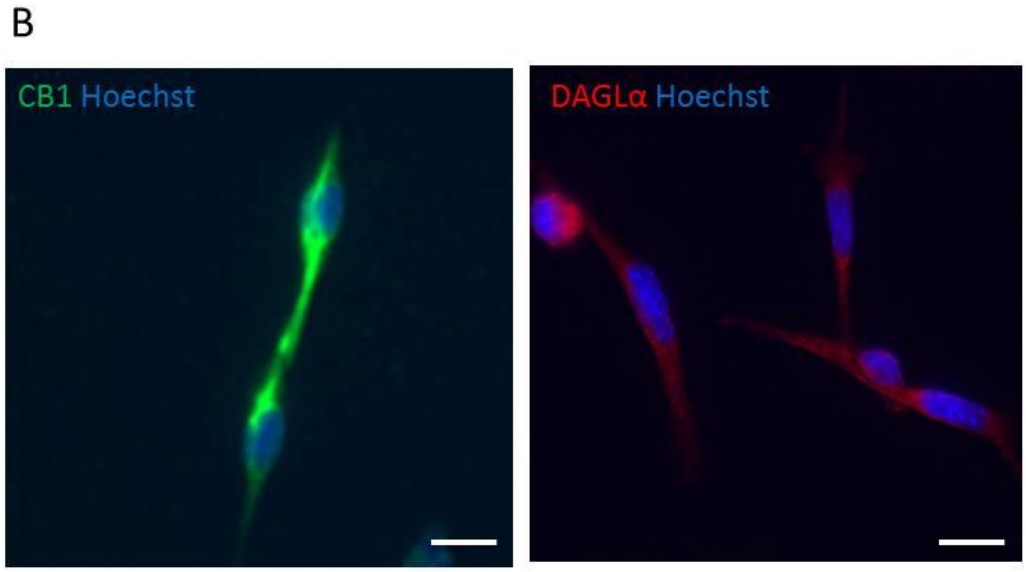


Figure 3.1: Cor-1 cells express neural stem cell markers and remain undifferentiated in culture

Cor-1 cells were cultured on gelatine coated glass coverslips in 4-well plates at 15,000 cells/well and left to attach overnight before being fixed with PFA and being used for immunocytochemistry. Hoechst was used to label the nuclei (A-F). Phalloidin staining was performed to visualize F-actin and the Cor-1 cell outline (A). Sox2 is a transcription factor which is important for self-renewal of embryonic stem cells and is shown in B. Nestin is a neural stem cell marker and is shown in C and D. Undifferentiated Cor-1 cells do not express the astrocyte marker GFAP (E) or neuronal lineage marker Tuj-1 (F). Scale bar in B, D, E and F 10µM.


A
Wt
DAGLβ KO
DAGLα KO
←DAGLβ (70kD)
← DAGLα (120kD)
← Actin


B
CB1 Hoechst
DAGLα Hoechst

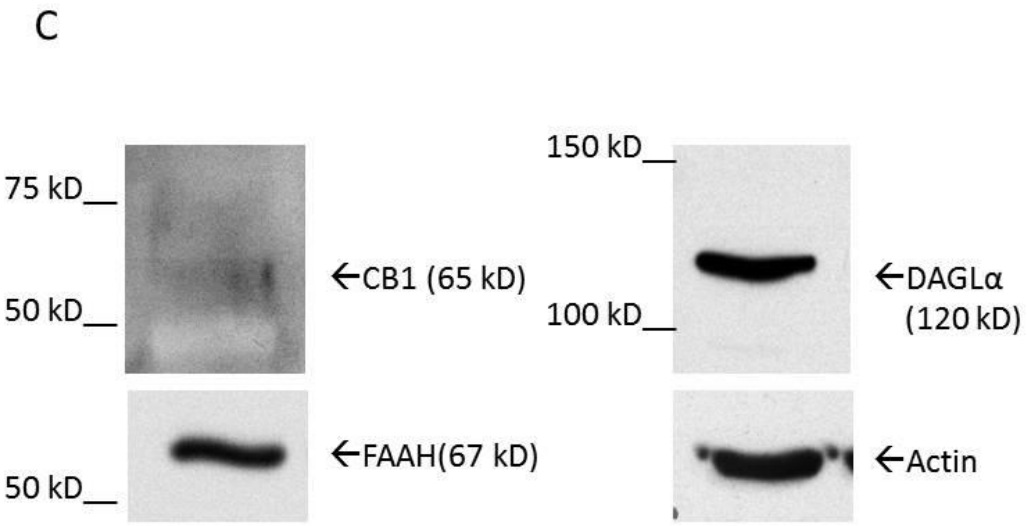

C
75 kD
50 kD
←CB1 (65 kD)
50 kD
←FAAH(67 kD)
150 kD
100 kD
←DAGLα
(120 kD)
←Actin

Figure 3.2: Validation of DAGLα/β antibodies and expression of various eCB system components by Cor-1 cells

Cerebellar tissue from wt mice, DAGLα KO mice or DAGLβ KO mice (kind gift from Pfizer) was used for western blot analysis. An anti-DAGLα antibody was used and identifies a 120 kDa band in the wt and DAGLβ tissue, but not the DAGLα tissue. DAGLβ antibodies detect a 70 kDa band in the wt and DAGLα tissue, but not DAGLβ KO tissue. Actin was used as a loading control and antibodies were detected using ECL Chemiluminescence detection (A). Cor-1 cells were grown on gelatine covered glass cover slips in 4-well plates at 15,000 cells/well and left overnight to attach before being fixed with PFA and an immunocytochemistry staining was performed. Anti-CB1 receptor and anti-DAGLα antibodies pick up the enzymes throughout the cell surface (B). Cor-1 cells were cultured in gelatine coated 6-well plates at a seeding density of 500,000 cells/well and left to attach overnight. The proteins were extracted and used for western blot analysis. Anti-CB1 receptor, anti-FAAH and anti-DAGLα antibodies recognised bands at the expected molecular weights. Actin was used as a loading control. Antibodies were detected using ECL Chemiluminescence detection (C). Scale bar in B: 10μM

In brief, 8,000 cells/well were seeded into a 96-well plate and left to attach overnight. The cells were treated with 0-1µM OMDM-188 for 48h and cell confluence was determined every 2h using the IncuCyte imaging platform. The cells looked healthy and had processes at all drug concentrations after the 48h treatment period (Figure 3.3 A). Representative growth curves are shown in Figure 3.3 B for a control culture and a culture treated with 0.5µM OMDM-188. The area under the curve was calculated for three independent experiments at the stated concentrations and normalised to the control. The relative area under the curve is shown Figure 3.3 C, demonstrating a significant reduction of ~25-35% in proliferation at 0.5 and 1µM OMDM-188. This data supports the hypothesis that DAGL dependent eCB signaling is required for Cor-1 (Goncalves et al., 2008).

3.3. Is eCB signaling required for Cor-1 cell differentiation?

3.3.1. Effects of eCB receptor antagonists and agonist on neuronal differentiation

A role for the eCB system in the proliferation and migration of Cor-1 and other NSC lines has been established (Goncalves et al., 2008; Oudin et al., 2011b). It is not known, if eCB signaling is required for the differentiation of Cor-1 cells to neurons or glia. I have differentiated Cor-1 cells towards a neuronal lineage using a B27 based neuronal differentiation protocol adapted from Conti et al. 2005, with an example of this shown in Figure 3.4. In brief, cells were grown on poly-ornithine and laminin coated 96-well plates in neuronal differentiation media for 7d with half the media being exchanged at day 4. During this time at least a subpopulation of the cells keeps proliferating. The media change causes some cells to wash off, which results in a dip in confluence and wider error bars after day 4 (Figure 3.4 B). By 4 days, the cells have developed more branches and longer processes which is indicative of adoption of a neuronal phenotype (Figure 3.4 A). How quickly Cor-1 cells display this morphology is highly dependent on initial seeding density and occurs quicker when they are seeded at a higher density.

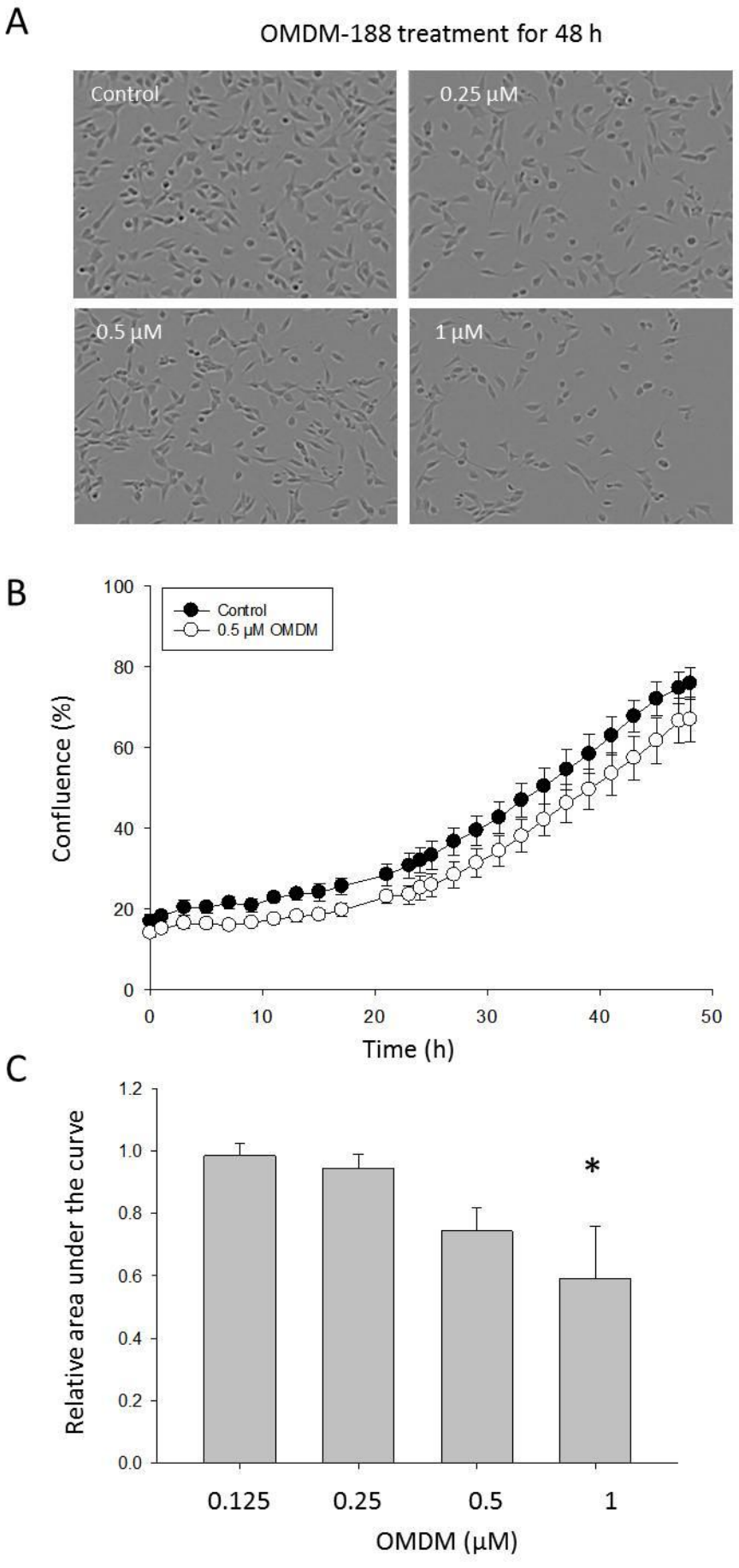

A
OMDM-188 treatment for 48 h
Control
0.25 µM
0.5 µM
1 µM
B
Control
0.5 µM OMDM
Confluence (%)
Time (h)
C
Relative area under the curve
OMDM (µM)
0.125
0.25
0.5
1
*

Figure 3.3: The DAGL inhibitor OMDM-188 reduces Cor-1 proliferation

Cor-1 cells were seeded at a density of 8,000 cells/well into 96-well plates and left overnight to attach. The cells were then treated with 1µM , 0.5µM or 0.25µM OMDM-188 and two phase-contrast pictures were taken every 2h for 48h using IncuCyte imaging. The cells looked healthy even after 48h of OMDM-188 treatment (A). Confluence of the cells was calculated using the IncuCyte software and a representative proliferation curve can be seen in B. The area under the curve was calculated using an Excel based Macro and results from 3 independent experiments were pooled. The data was normalised to the control and the relative mean ± SEM is displayed. Anova analysis (one way analysis of variance) was carried out conforming significance between treatment groups ($p=0.014$). Post-hoc testing (Tukey test) revealed a significance ($p=0.023$) between the control and 1µM OMDM.

(C):* $p<0.05$, bars show mean ± SEM

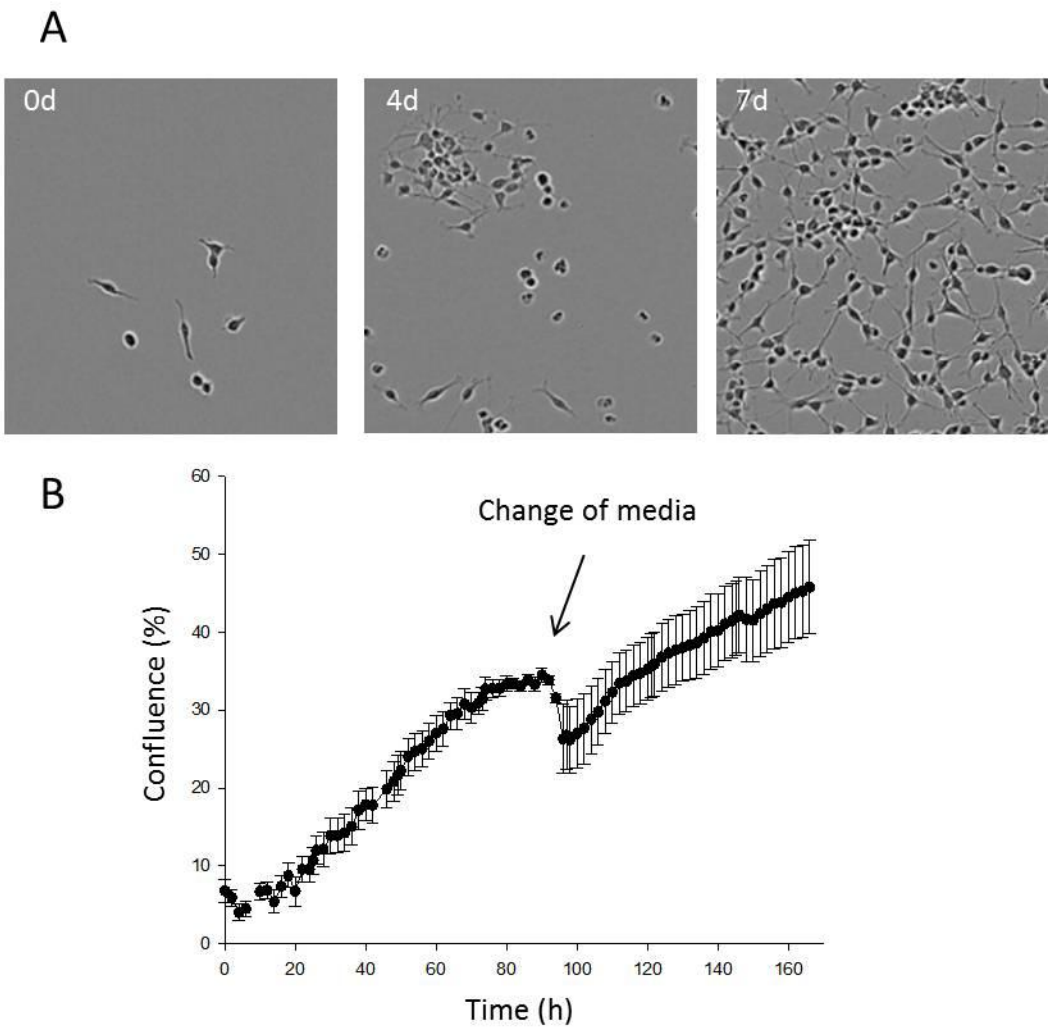


Figure 3.4: Differentiation of Cor-1 cells with a neuronal differentiation protocol for 7 d

Cor-1 cells were seeded at 3,000 cells/well into 96-well plates coated with poly-ornithine and laminin. After the cells were left to attach overnight the media was changed to the neuronal differentiation media for 7d and half the media was exchanged on day 4. The proliferation was followed using the IncuCyte platform with images being taken every 2h for 48h. Pictures shown are from days 0, 4 and 7d of differentiation. Cells have longer and branched processes at 4d and 7d of differentiation (A). Confluence is presented against time and data was pooled from 6 wells (B).

The effect of CB1 and/ or CB2 receptor antagonist on neuronal Cor-1 cell differentiation was investigated. Cor-1 cells were seeded at 20,000 cells/well in 4-well plates onto poly-ornithine and laminin coated cover slips and left to attach overnight in expansion media. The next day, the media was replaced with differentiation media as well as 1µM of a CB1 receptor antagonist (AM251) and/or a CB2 receptor antagonist (JTE-907). After 2d half the media was replaced by fresh neuronal differentiation media containing fresh drugs. The cells were grown for two more days and then fixed and stained for the neuronal lineage marker Tuj-1 as well as the fluorescent DNA binding dye Hoechst. Tuj-1 positive cells as well as the total cell number were counted, and the % of Tuj-1 positive cells to total cell number is presented in Figure 3.5 E. Cells were only counted if in focus and not in a cell cluster. 3 cover slips per condition were used for the analysis and the average of 3 independent experiments is presented. On average, 20% of all cells are Tuj-1 positive. This is not changed by CB1 and/or CB2 receptor antagonism under the observed conditions. The same experimental setup was also used to establish the effect of CB1 and/or 2 receptor agonists 1µM ACEA and 1µM JWH-133 on neuronal differentiation (Figure 3.6). Neither CB1 nor CB2 receptor agonism changed the % of Tuj-1 positive cells to total cell number. Combined these results show that neither CB1 and/or CB2 receptor antagonism nor CB1 or CB2 receptor agonism have an influence on the differentiation of Cor-1 cells towards the neuronal lineage. Thus the eCB signaling pathway is most likely not required for neuronal differentiation, nor does it stimulate it when activated in Cor-1 cells.

3.3.2. The effects of eCB antagonists on astrocyte differentiation

Cor-1 cells can also be differentiated to astrocytes using a BMP-4 or goat serum based protocol (adapted from Conti *et al*. 2005). Cor-1 cells were seeded at 5,000 cells/well onto gelatine coated 96-well plates, left to attach overnight, and the media was exchanged to BMP-4 astrocyte differentiation media. The morphology of the cells was observed for 7d using IncuCyte live cell imaging. At day 0, the cells display their cell typical polar morphology, but at day 4 a more star like morphology can be seen for most cells which is typical for astrocytes. The cell body appears more spread out and flatter.

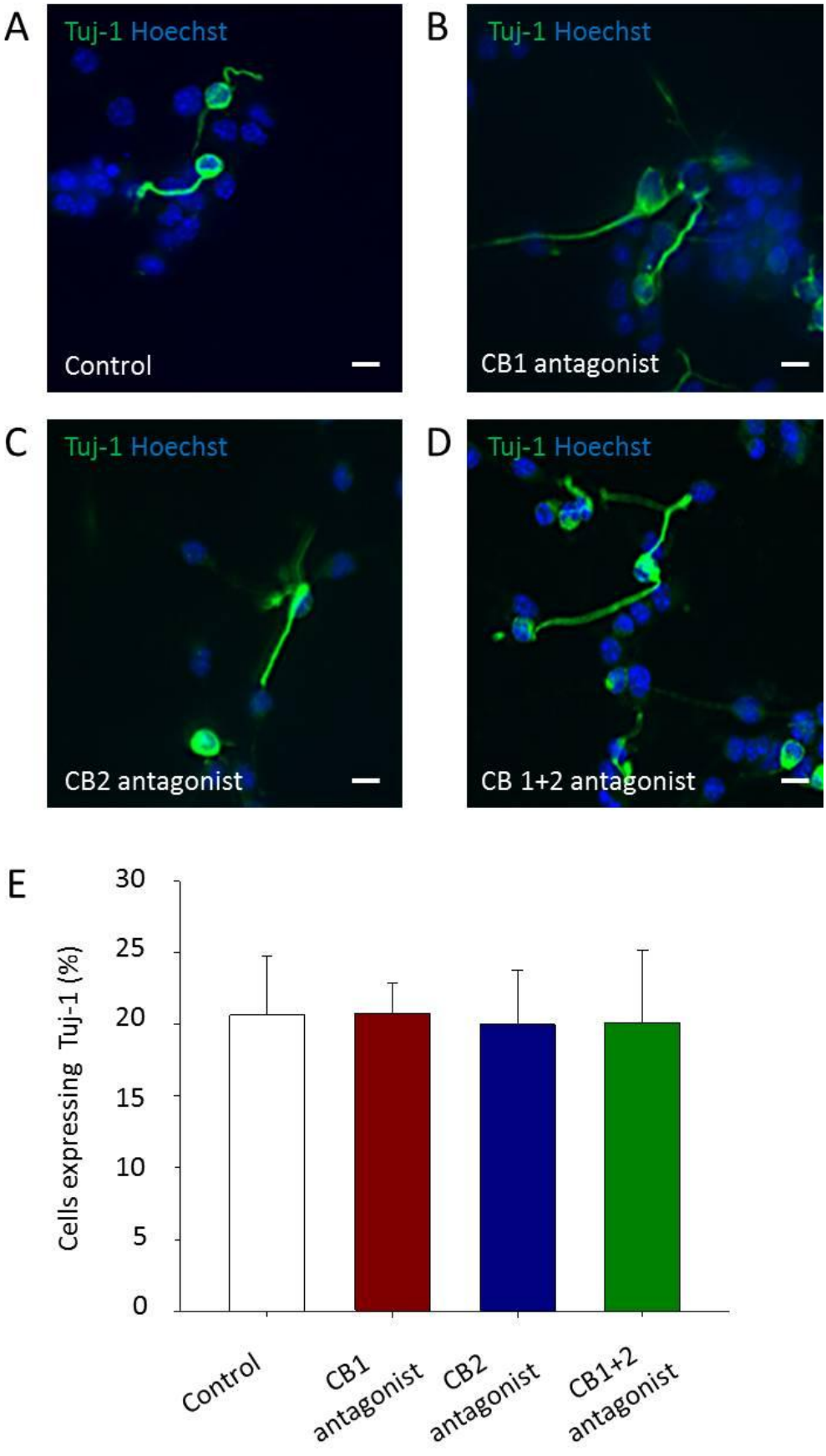

A
Tuj-1 Hoechst
Control
B
Tuj-1 Hoechst
CB1 antagonist
C
Tuj-1 Hoechst
CB2 antagonist
D
Tuj-1 Hoechst
CB 1+2 antagonist
E
Cells expressing Tuj-1 (%)
30
25
20
15
10
5
0
Control
CB1 antagonist
CB2 antagonist
CB1+2 antagonist

Figure 3.5: Effect of CB1 and/or 2 receptor antagonist treatment on neuronal differentiation of Cor-1 cells

20,000 Cor-1 cells were seeded onto poly-ornithine and laminin coated cover slips in 4-well plates and grown in expansion media overnight. The media was changed to neuronal differentiation media and the cells were grown for a total of 4d under these conditions. A subset of cells was additionally treated with either 1µM AM251 (CB1 receptor antagonist) and/or 1µM JTE-907 (CB2 receptor antagonist). Half the media was changed for fresh neuronal differentiation media after 2d of differentiation, maintaining a steady concentration of AM251 and/or JTE-907. Afterwards the cells were fixed using PFA and stained for the neuronal lineage marker Tuj-1 and fluorescent DNA binding substance Hoechst (A-D). The number of Tuj-1 positive cells per total cell number was counted for the control as well as for the samples treated with the CB1 and/or 2 receptor antagonists. The percentage of Tuj-1 positive cells is presented. Three coverslips were counted per experiment and the bar shows the mean of three independent experiments ± SEM (E). Scale bar in A-D: 10µM.

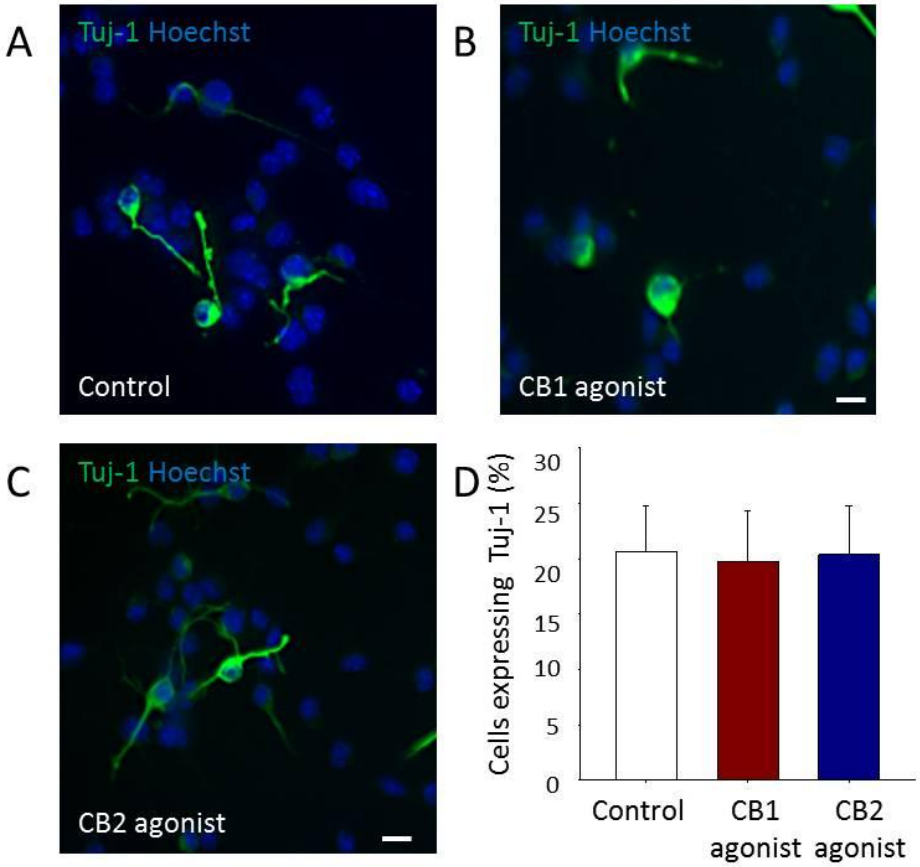


Figure 3.6: Effect of CB1 and/or CB2 receptor agonist treatment on neuronal differentiation of Cor-1 cells

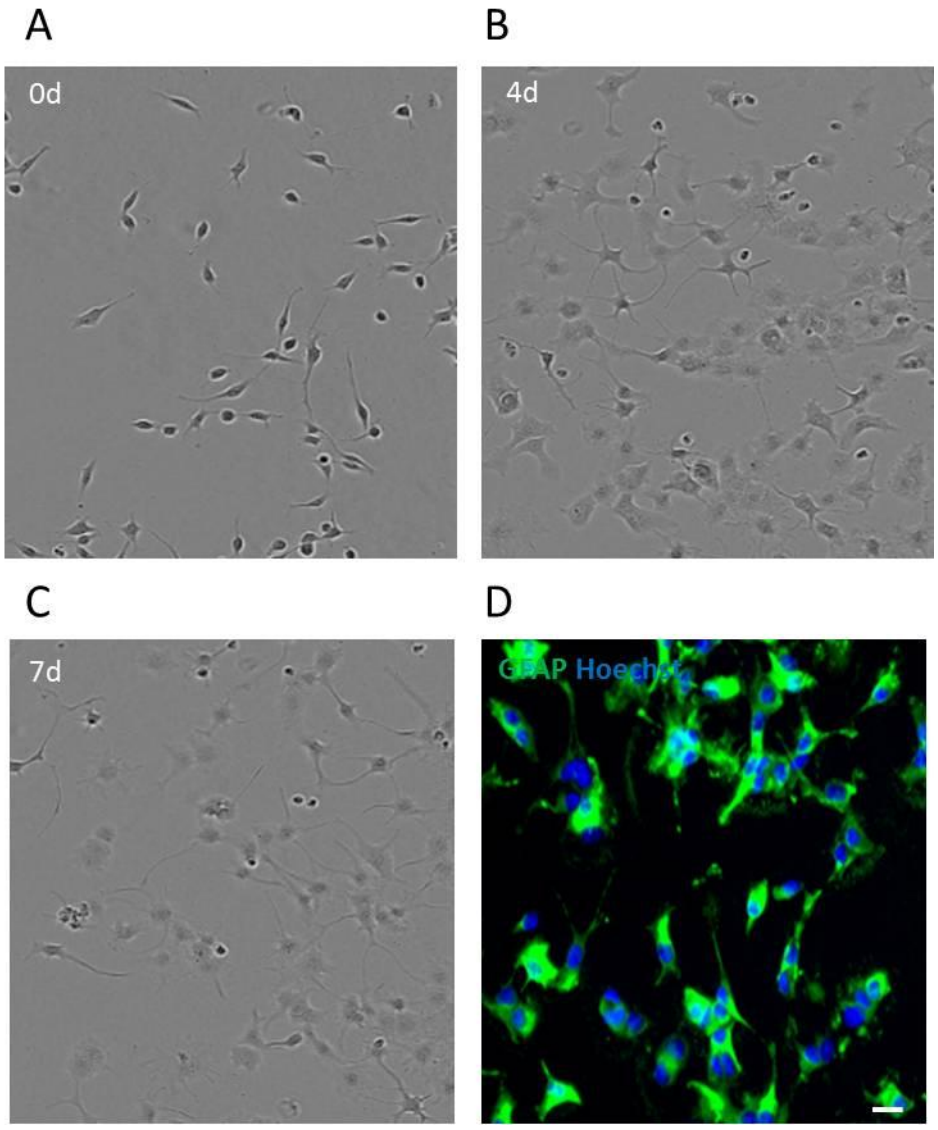
20,000 Cor-1 cells were seeded onto poly-ornithine and laminin coated cover slips in 4-well plates and grown in expansion media overnight. The media was changed to neuronal differentiation media for 4d. A subset was additionally treated with either 1µM ACEA (CB1 receptor agonist) or JWH-133 (CB2 receptor antagonist). Half the media was changed for fresh neuronal differentiation media after 2d maintaining a steady concentration of AM251 or JTE-907. Afterwards the cells were fixed and stained for the neuronal lineage marker Tuj-1 and fluorescent DNA binding substance Hoechst (A-C). Tuj-1 positive cells per total cell number were counted on 3 coverslips per experiment. The percentage of Tuj-1 positive cells is presented as the mean of 3 independent experiments ± SEM. Scale bar in B and C: 10µM.

The pictures of day 0, 4, and 7 show the same picture frame. Even though cells do not seem to proliferate anymore, they remain mobile and move around (Figure 3.7 A-C). Undifferentiated Cor-1 cells do not express GFAP (Figure 3.1). To analyse how many Cor-1 cells express the astrocytic marker GFAP after astrocytic differentiation, 20,000 Cor-1 cells/well were seeded into 4-well plates containing gelatine coated glass cover slips, differentiated for 4d using a BMP-4 based astrocyte differentiation protocol and fixed. The cells were stained with the DNA dye Hoechst and the astrocytic differentiation marker GFAP. 98% of all cells express GFAP and a representative picture is shown in Figure 3.7 D.

To establish the effect of CB1 and/or CB2 receptor antagonism, 20,000 Cor-1 cells were seeded onto gelatine coated glass cover slips and left to attach overnight in expansion media and were then differentiated for 24h using a BMP-4 based protocol (Figure 3.8). A subset was also treated with 1µM CB1 (AM251) or 1µM CB2 (JTE 907) receptor antagonists, the cells were fixed and stained with Hoechst, GFAP and Phalloidin. Phalloidin is an F-Actin marker, used here to visualise the cell morphology and representative pictures are shown (Figure 3.8 A-C). The BMP-4 based protocol yields ~ 30% GFAP positive cells after 1d of differentiation (Figure 3.8 D) and the CB1 and/or CB2 receptor antagonists had no effect on the percentage of GFAP positive cells. This demonstrates that eCB signaling is not required for Cor-1 cell differentiation to an astrocyte lineage and similar results were obtained using the "goat-serum" based astrocytic differentiation protocol (results not shown).

3.4. Characterization of DAGLα/β transfected cell lines

The above results indicate that the eCB system does not play a role in the differentiation of Cor-1 cells to neurons or astrocytes. We were interested to know if there might be a change in the expression and/or localisation of the DAGLs between the various lineages. To this end we wanted to follow the endogenous DAGLs and epitope tagged versions of the DAGLs.


A
B
0d
4d
C
D
7d
GFAP Hoechst

#

Figure 3.7: Differentiation of Cor-1 cells with an astrocytic differentiation protocol

Cor-1 cells were seeded at 5,000 cells/well into a gelatine coated 96-well plate. The cells were left to attach overnight, followed by differentiation with an BMP-5 based astrocytic differentiation media for 7d and proliferation was observed using IncuCyte™ live cell imaging. Pictures shown are from days 0, 4 and 7 of differentiation and cells show an astrocytic phenotype at 4d and 7d (A-C). 20,000 Cor-1 cells were seeded into 4-well plates containing gelatine coated cover slips and differentiated with a BMP-4 based astrocytic differentiation protocol for 4d, fixed and stained with the DNA dye Hoechst and the astrocytic marker GFAP (D). Scale bar in D: 10µM.

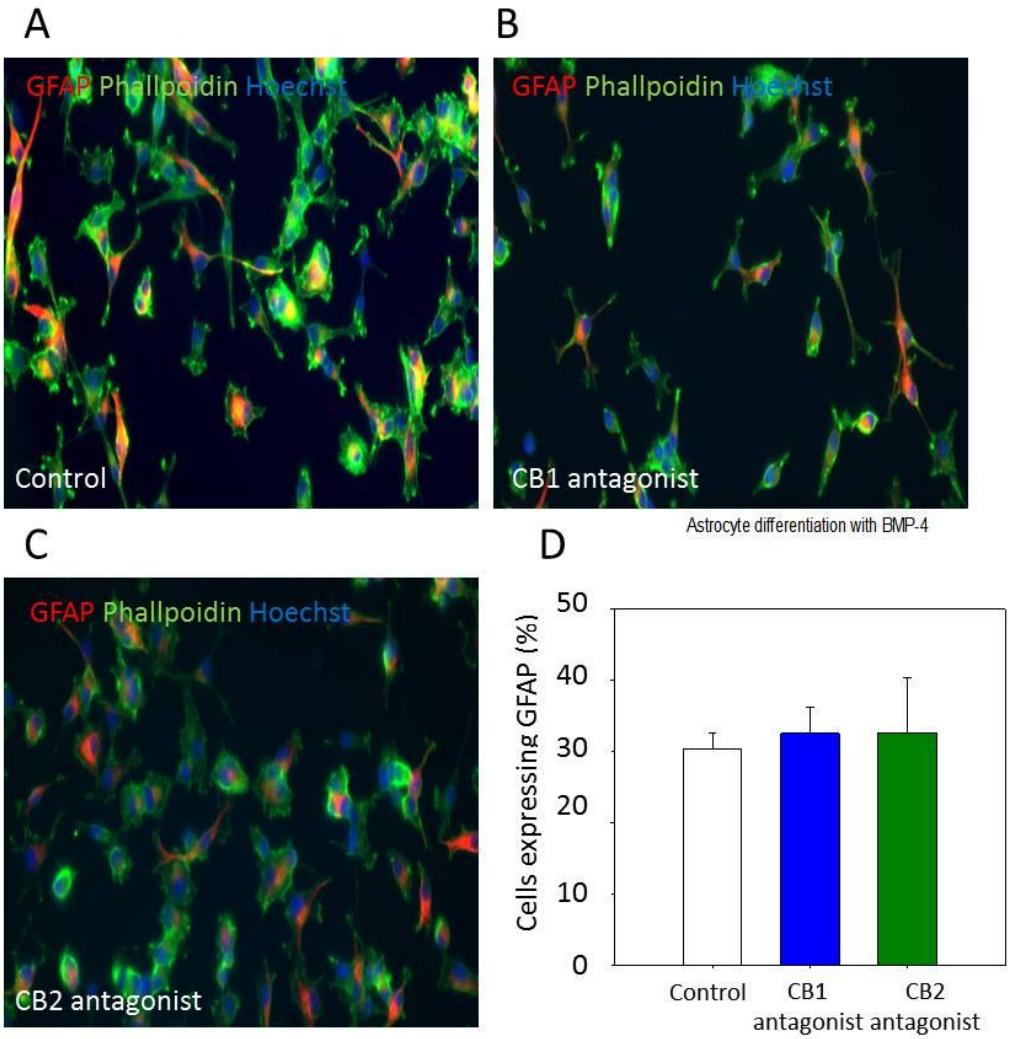


Figure 3.8: Astrocytic differentiation of Cor-1 cells under CB1/2 receptor antagonist treatment

20,000 Cor-1 cells were seeded onto gelatine coated cover slips in 4-well plates and differentiated for 24h using the BMP-4 based astrocytic differentiation protocol. A subset was additionally treated with either 1µM AM251 (CB1 receptor antagonist) or JTEC 907 (CB2 receptor antagonist). Cells were fixed and stained for GFAP as an astrocytic marker, Phalloidin and Hoechst (A-C). GFAP positive cells as well as the total cell number were counted on 3 coverslips per experiment and the percentage of GFAP positive cells is displayed in D as the mean of three independent experiments ± SEM.

Cor-1 cell lines which overexpress DAGLα or β were established and made available for the study (provided by Dr. Philipp Sütterlin, Doherty lab). The cell lines express an epitope-tagged DAGLα or DAGLβ under the CMV promoter and were named V5α7 and V5β5 respectively.

Western blot analysis was performed to confirm expression of DAGLα-V5 and DAGLβ-V5 as well as to establish relative expression levels (Figure 3.9). V5α7 and V5β5 cells were expanded in growth media and their proteins extracted and subjected to western blot analysis. In this context, an anti-V5 antibody was applied and visualised using ECL. V5α7 cells express the 120 kDa DAGLα-V5 construct. A second band can be at about 260 kDa, suggesting the existence of a higher order DAGLα complex. Between the bands seen at 120 and 260 kDa a diffuse staining can be seen, which might point towards glycosylation of DAGLα-V5. It has to be mentioned, that using an endogenous anti-DAGL antibody, only one band at 120 kDa was seen (Figure 3.2). A single band at 70 kDa is seen in the V5β5 cells, which is the expected molecular weight for DAGLβ (Bisogno et al., 2003). The expression of DAGLβ is considerably lower than the DAGLα expression seen in the V5α7 cells.

The V5α7 and V5β5 cell lines were further characterized using immunocytochemistry (Figure 3.10). 20,000 cells were grown on gelatine coated cover slips, left to attach in expansion media overnight, fixed and used for immunocytochemistry. Both cell lines continue to express the NSC marker nestin (Figure 3.10 A+B) and express the corresponding construct throughout the whole cell population. No positive staining can be seen when native Cor-1 cells are stained with an anti-V5 antibody (data not shown). DAGLα-V5 appears to be localized on the cell surface within the V5α7 cells (Figure 3.10 C+D), while DAGLβ-V5 staining Figure 3.10 E+F) is punctuated and possibly retained to some extent within the endoplasmic reticulum. We decided to take the DAGLα transfected cells forward for additional study based on the higher level of expression relative to the transfected DAGLβ cells.

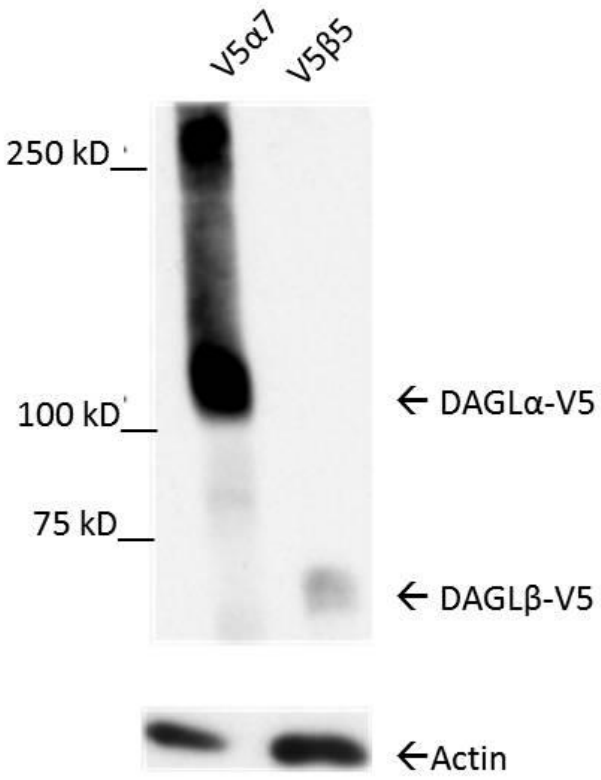


Figure 3.9: DAGLα-V5 or DAGLβ-V5 are expression in V5α7 and V5β5

Stable Cor-1 cell lines expressing the epitope-tagged DAGLα or DAGLβ were created and named V5α7 and V5β5, respectively. The cells were expanded, the protein was extracted and used at equal amounts for western blot analysis. An anti-V5 antibody was used to detect DAGLα-V5 in the V5α7 cells and DAGLβ-V5 in the V5β5 cells. This confirms the expression of DAGLα-V5 at 120 kDa (V5α7) and DAGLβ-V5 (V5β5) at 70 kDa. Actin was used as a loading control. An ECL detection kit was used to visualize the antibodies.

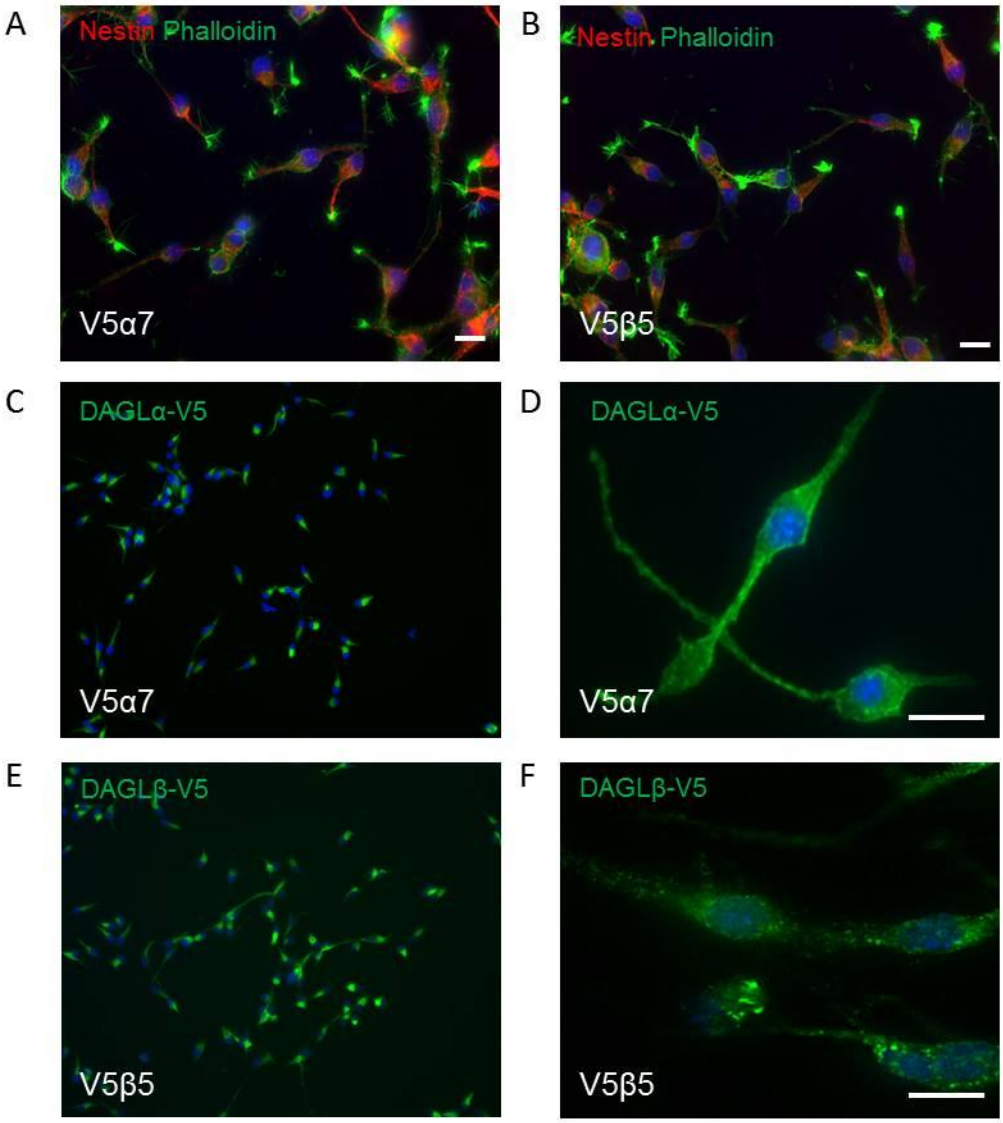


Figure 3.10: DAGLα-V5 (V5α7) or DAGLβ-V5 (V5β5) overexpressing Cor-1 cells

20,000 V5α7 or V5β5 were seeded onto gelatine coated cover slips in 4-well plates, left to attach in expansion media overnight and fixed with PFA. An anti-nestin antibody was used to detect this neural stem cell marker. Phalloidin was used to visualise the cell morphology (A+B). An anti-V5 antibody was used to detect the epitope tagged DAGL constructs expressed by V5α7 and V5β5 cells (C+F). Scale bars in A,B,D,F : 10µM

The morphology of Cor-1 and V5α7 was compared using immunocytochemistry. The cells were grown on gelatine coated glass coverslips at 20,000 cells/well in 4-well plates and left in expansion media to attach overnight, fixed using PFA and a phalloidin dye was used to visualize the cell shape. The Axio Vision LE imaging software was used to measure cell area (Figure 3.11 A) and process length (Figure Figure 3.11 C) and process number/cell (Figure 3.11 B) manually. The cell area of Cor-1 cells is approximately 250μm^2 and V5α7 cells have a tendency to have a smaller cell area of approximately 240μm^2 however this is not a significant difference. Both cell types have on average 2 processes, which have an average length of 23μm. Thus overexpression of DAGLα is not obviously detrimental to the cells' morphology.

3.5. DAGLα expression during Cor-1 differentiation

3.5.1. DAGLα levels during neuronal differentiation

The expression of DAGLα was examined in Cor-1 and V5α7 cells during differentiation to a neuronal phenotype. Cor-1 cells were differentiated for 0-4 d with the neuronal differentiation protocol before protein extraction and western blot analysis. An anti-DAGLα antibody was used to monitor DAGLα expression and was visualized using ECL and the result is shown in Figure 3.12. Furthermore, Tuj-1 is used to monitor the differentiation towards a neuronal lineage, which was expressed from day 3 onwards and actin served as a loading control. A very substantial decrease in DAGLα expression was observed over the course of differentiation. On neuronal differentiation day 1-3 only a faint band can be detected with the anti-DAGLα antibody and it is almost entirely absent by day 4.

As is shown in Figures 3.13, only a proportion of cells (20-30%) express Tuj-1, when they are differentiated towards neurons. To determine if astrocytes are also generated in the neuronal differentiation protocol, protein extracts were also analysed by western blot for the appearance of GFAP. At day 1 of differentiation the astrocytic marker GFAP is also starting to be expressed, with expression further increasing on day 2 and 3 confirming the presence of astrocytic cells (Figure 3.12 B).

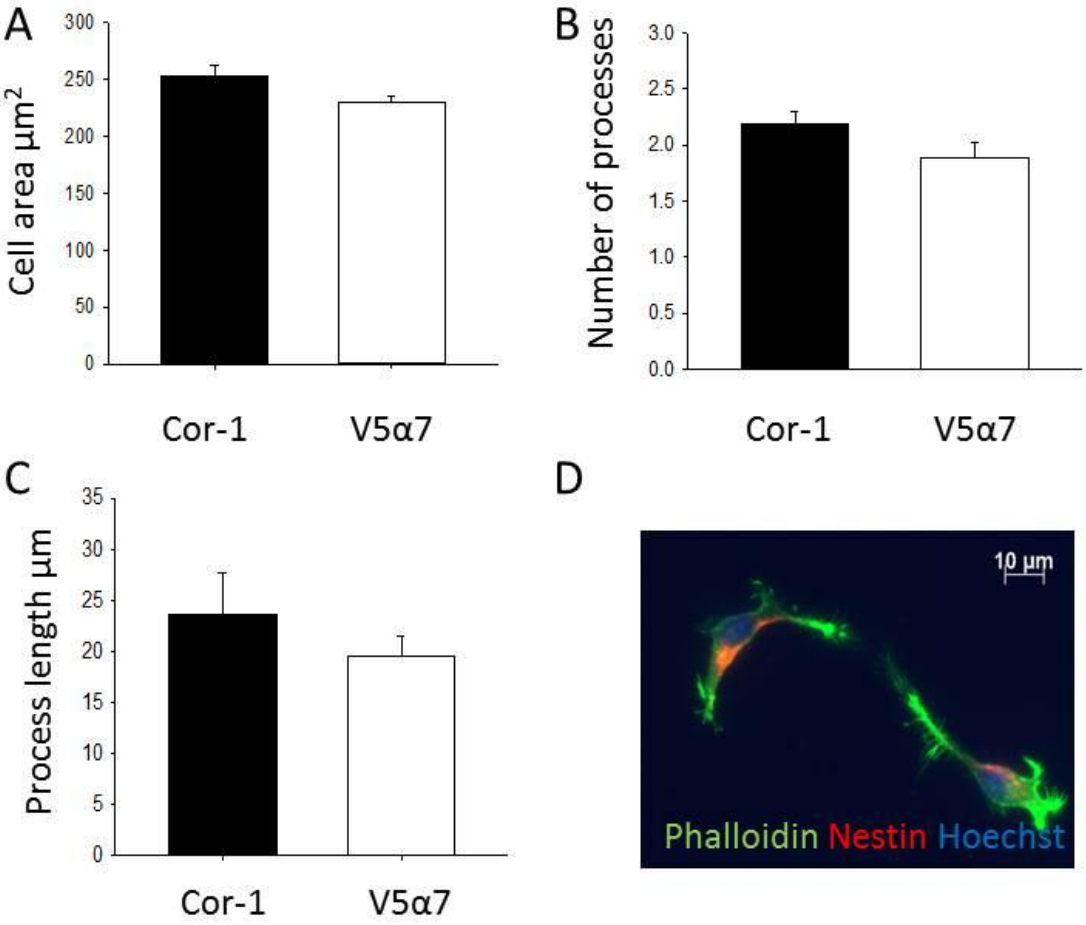


Figure 3.11: Morphology comparison of Cor-1 and V5α7 cells

Cor-1 and V5α7 stained with Phalloidin, Nestin and Hoechst. The cell area (A) and process length (C) were measured using the Axio Vision LE software (Zeiss). The process number was counted manually. A representative picture of Cor-1 cells are shown in D. No significant differences were observed. All graphs display the mean of three independent experiments ± SEM. No significant difference was observed (student t-test).

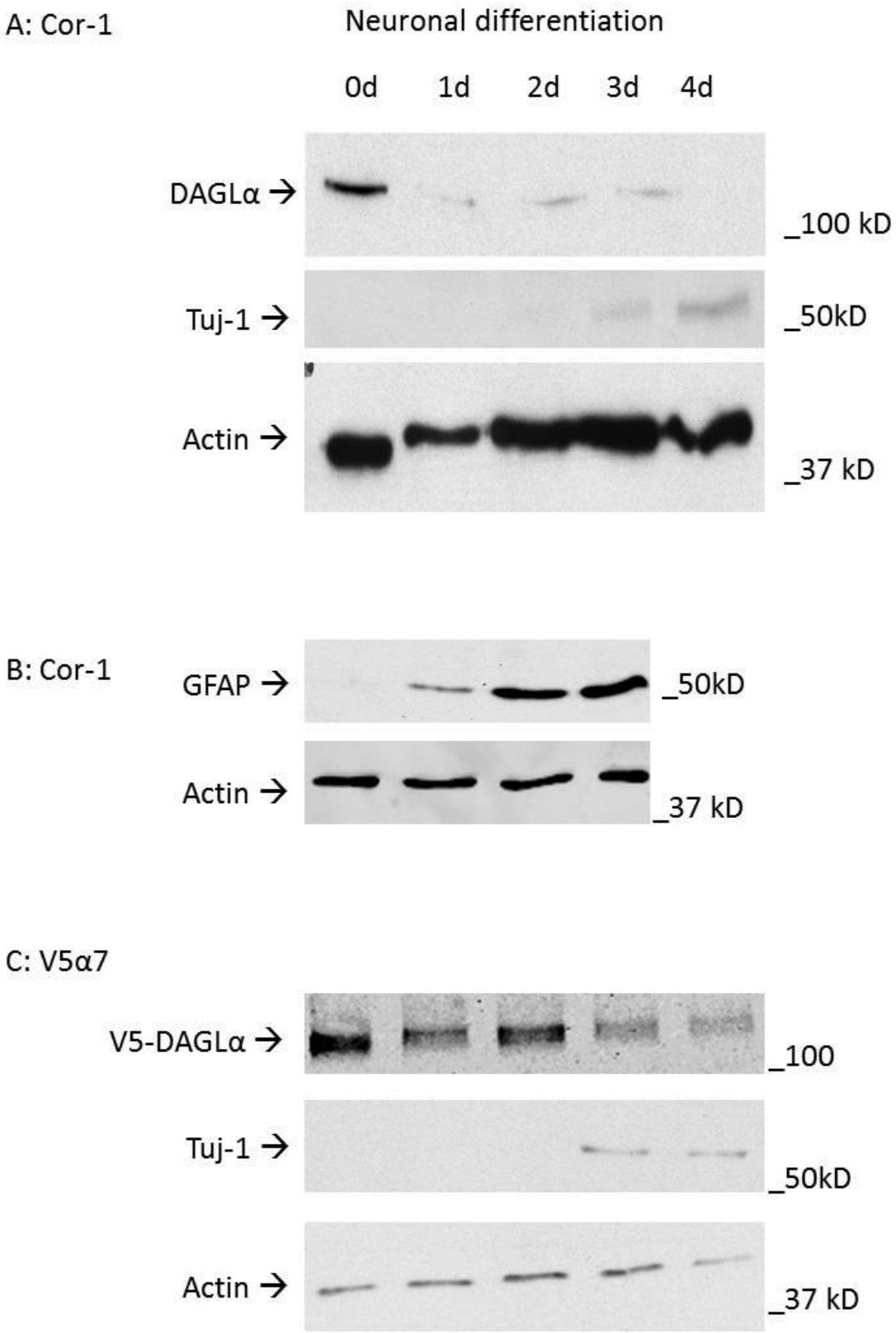

A: Cor-1
Neuronal differentiation
0d
1d
2d
3d
4d
DAGLα →
_100 kD
Tuj-1 →
_50kD
Actin →
_37 kD
B: Cor-1
GFAP →
_50kD
Actin →
_37 kD
C: V5α7
V5-DAGLα →
_100
Tuj-1 →
_50kD
Actin →
_37 kD

Figure 3.12: Overexpressed DAGLα-V5 as well as endogenous DAGLα expression is decreased during differentiation of Cor-1 cells with a neuronal differentiation protocol

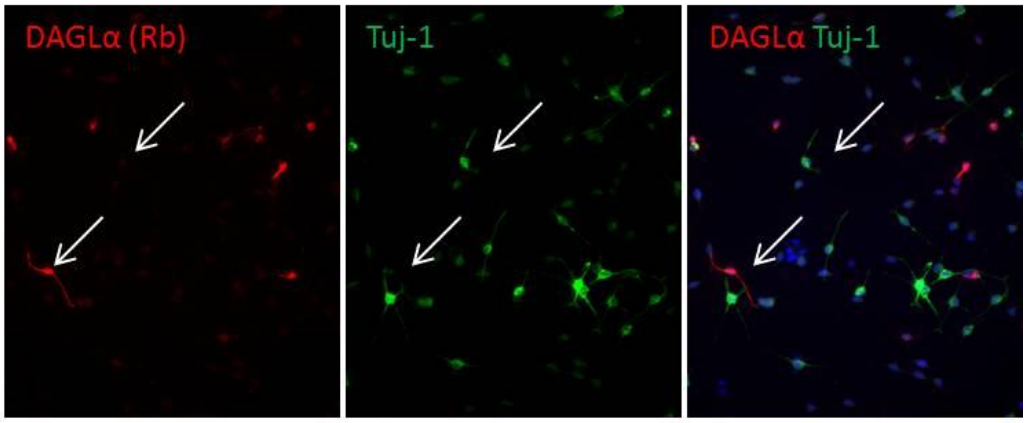
Cor-1 cells were differentiated for 0-4d with the neuronal differentiation protocol before protein extraction. The samples were used for western blot analysis. An anti-DAGLα antibody was used to monitor DAGLα expression, an anti-Tuj-1 antibody was used to observe neuronal differentiation and actin was used as a loading control. The antibodies were visualized using ECL (A). Cor-1 cells were differentiated for 0-3d with the neuronal differentiation protocol, the proteins were extracted and used for western blot analysis. An anti-GFAP antibody was used to monitor the generation of astrocytic cells and ECL was used for visualization. Actin serves as a loading control (B). V5α7 cells were differentiated for 0-4d with the neuronal differentiation protocol before protein extraction and western blot analysis. An anti-V5 epitope antibody was used to monitor DAGLα –V5 expression and was visualized using ECL. Actin served as a loading control (C).

Therefore a mixed population of cells arises from Cor-1 cells using the neuronal differentiation protocol leading to an interest in the DAGLα expression levels in Cor-1 cells differentiated upon astrocyte formation. We moreover wanted to know if transfected DAGLα-V5 is regulated in the same way as the endogenous DAGLα, as this would suggest regulation at the level of protein expression rather than solely at the level of transcription.

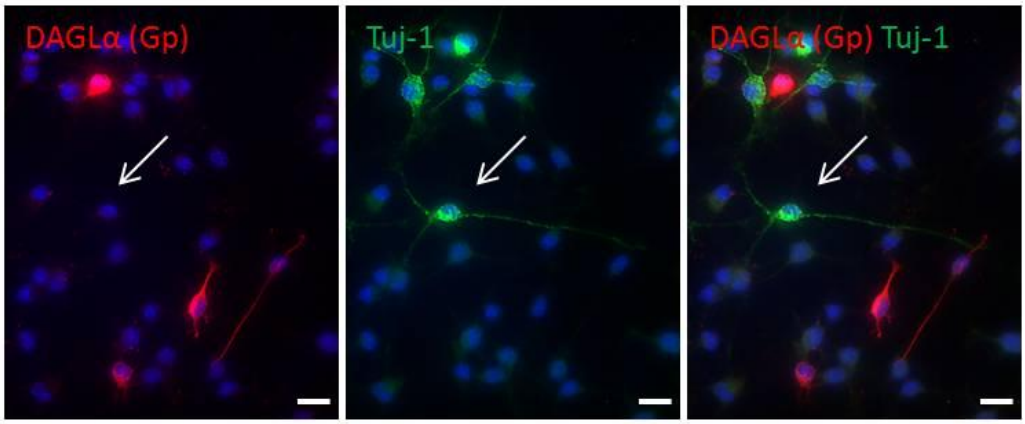
V5α7 cells were differentiated for 0-4d as described above; their protein was extracted and used for western blot analysis. An anti-V5 antibody was used to detect DAGLα-V5 and a decrease in the expression is also seen, albeit at a slower rate than that observed for the endogenous DAGLα (Fig 3.12 C). Tuj-1 expression was again used to confirm neuronal differentiation during this period and actin was used as a loading control. When comparing DAGLα/DAGLα-V5 expression in Cor-1 and V5α7 cells, a decrease in expression upon neuronal differentiation is observed in both cases. This demonstrates that overall endogenous as well as overexpressed DAGL expression levels are reduced, and this suggests regulation by a means other than simply gene transcription (unless there is a coincidental loss of the different factors driving the endogenous and transgenic genes).

Our results show that neurons and astrocytes are generated when Cor-1 cells are switched from their normal growth conditions to the "neuronal" differentiation conditions. We wanted to test if DAGLα expression is down-regulated when Cor-1 cells become neuronal. To do this, Cor-1 or V5α7 cells were differentiated for 7d using the neuronal differentiation protocol and cell-type composition determined by immunocytochemistry (Figure 3.13). Compared to the neuronal differentiation used for western blot analysis (Figure 3.12) a longer neuronal differentiation protocol was used for this experiment. This allowed the initial low number of cells to spread out evenly. Anti-DAGLα antibodies were used to test for co-expression in Tuj-1 positive cells (Figure 3.13 A+B). The results clearly show that DAGLα expression is strongly reduced in Tuj-1 positive cells. Similarly, DAGLα-V5 expression was also significantly decreased in Tuj-1 positive V5α7 cells (Figure 3.13 C). Thus we can conclude that one feature of differentiation of Cor-1 cells to a neuronal phenotype is a rapid down-regulation of DAGLα expression.

A: Cor-1



B: Cor-1



C: V5α7

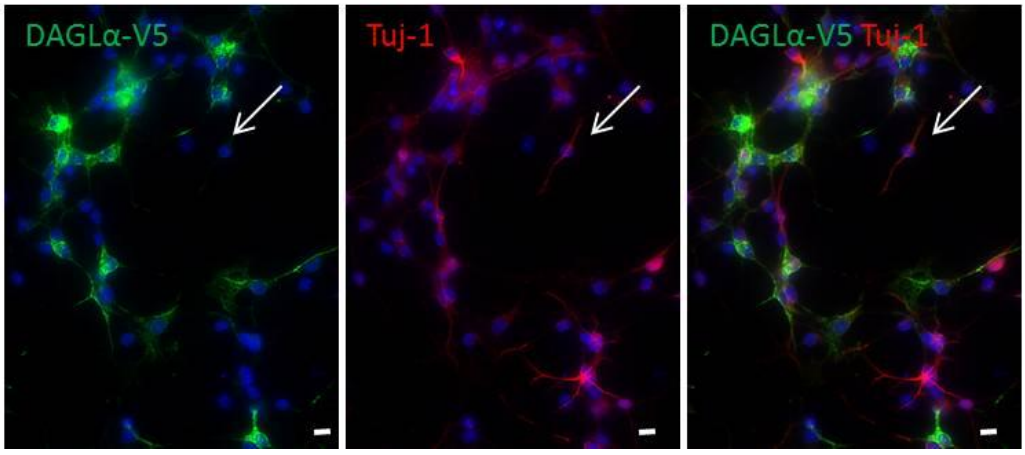


Figure 3.13: Differentiation of Cor-1 or V5α7 cells with a neuronal differentiation protocol for 4 d

Cor1 cells were differentiated for 4d using a neuronal differentiation protocol on poly-ornithine and laminin coated glass coverslips. The cells were fixed and used for immunocytochemistry. Two different anti-DAGLα antibodies were used to detect the enzyme. (A) An anti-DAGLα (rabbit, Watanabe) and (B) anti-DAGLα (guinea pig, Watanabe) were used in combination with anti-Tuj-1 (A+B) . The same experimental set up was used to differentiate V5α7 cells toward a neuronal phenotype for 4d. The cells were fixed and used for immunocytochemistry. An anti-V5 antibody was used to detect DAGLα-V5 in combination with anti-Tuj-1 antibody (C). Arrows point out the rapidly reduced DAGLα-V5 expression in cells expressing Tuj-1 in processes. Scale bar in B+C: 10µM.

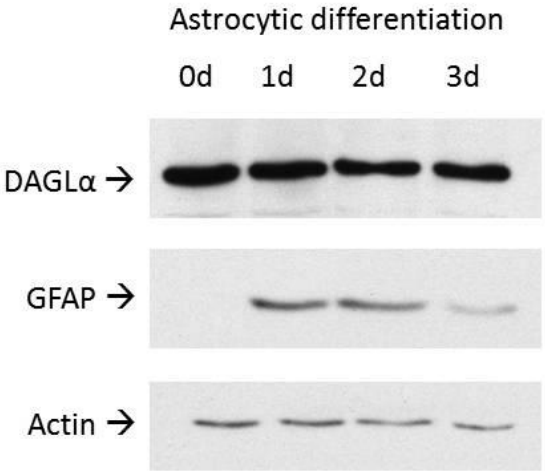
Considering that the endogenous enzyme and the overexpressed enzyme are expressed under different promoters, it is likely that the observed reduction in DAGLα expression levels seen in Figure 3.12 and 3.13 is related to regulation at the protein level. In support of this we have been unable to detect any changes in DAGLα mRNA levels in undifferentiated and differentiated cultures of Cor-1 cells (Doherty lab, unpublished observation). However, the mixed nature of the cell types found in the differentiated cultures complicates the picture and at this stage we cannot draw firm conclusions.

3.5.2. DAGLα levels during astrocyte differentiation

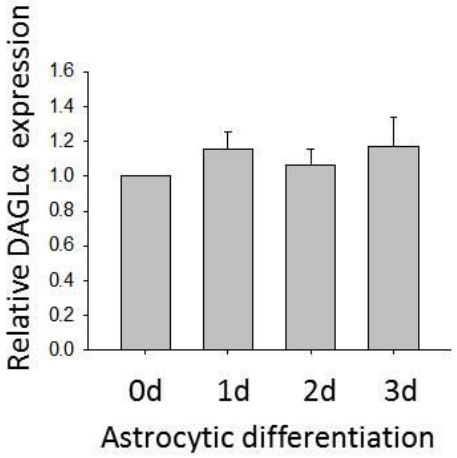
To determine if endogenous DAGLα levels changed during differentiation to astrocytes, Cor-1 cells were grown in the BMP-4 based astrocytic differentiation media for up to 3d before the proteins were extracted from the cells and analysed by western blotting. A comparison of DAGLα and GFAP expression over the 3d period is shown in Fig 3.14 A alongside the actin loading control. The band intensity was also measured using the Image-J software and DAGLα expression relative to actin is presented in Figure 3.14 B. In contrast to the very rapid increase in GFAP, there is no change in the relative level of endogenous DAGLα over the 3d period.

Next we wanted to determine if the expression levels of transfected DAGLα-V5 in V5α7 cells is changed during differentiation to astrocytes, and performed the same experiment as described above using the V5α7 cells. In contrast to the result observed for Cor-1 cells where DAGLα expression remained unchanged, we observe a rapid up-regulation of DAGLα-V5 (Fig 3.14 C+D). This result was unexpected, and as we did not see the same response with endogenous DAGLα, we have not explored the basis of it further. This effect is most likely caused by enhanced activation of the CMV promoter in differentiated astrocytes, which drives the expression of the transfected DAGLα-V5. The CMV promoter is a widely used promoter for transgene expression, but efficacy vary across different cells and is known to be affected by embryonic differentiation (Chung et al., 2002; Zeng et al., 2003; Bagchi et al., 2006; Hong et al., 2007).

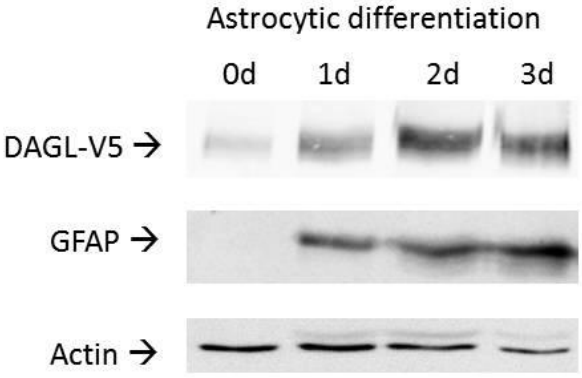
A:Cor-1



B:



C:V5α7



D:

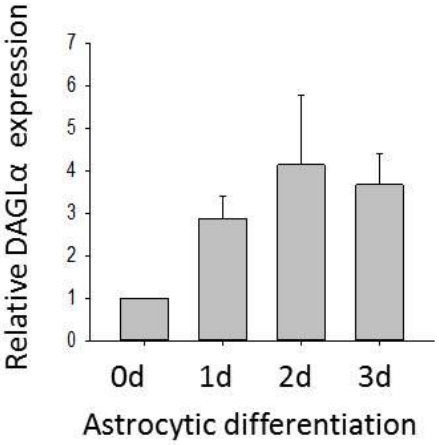


Figure 3.14: Endogenous DAGLα(-V5) expression during differentiation of Cor-1 cells to astrocytes

Cor-1 (A+B) or V5α7 cells (C+D) were differentiated for 0-3d with the astrocytic differentiation protocol before protein extraction. The samples were used for western blot analysis. The bands were quantified making use of the built-in Odyssey quantification tool. An anti-DAGLα antibody was used to monitor endogenous DAGLα expression in Cor-1 cells. The anti-GFAP marker is used to monitor the differentiation to astrocytes. Actin serves as a loading control (A). The expression levels of the DAGLα band in relation to actin was measured as described above and normalised to expression on day 0 (B). An anti-V5 epitope antibody was used to monitor the overexpressed DAGLα-V5 in V5α7 cells. The anti-GFAP marker is used to monitor the differentiation to astrocytes and actin serves as a loading control (C). The expression levels of the DAGLα band in relation to actin was measured as described above and normalised to expression on day 0 (D). B and D display the mean of three independent experiments ± SEM.

3.6. Conclusions and discussion

We have used the Cor-1 cell line as a model to address questions relating to the role of eCB signaling in the proliferation and differentiation of these cells to neurons and astrocytes. The Cor-1 cells line can be continuously expanded while maintaining certain stem cell characteristics. They have a doubling time of approximately 24h in our hands and keep expressing neuronal stem cell markers such as Sox2 and nestin (Figure 3.1) as has been described before (Conti et al., 2005). Moreover, they can be differentiated into astrocytes, neurons, and oligodendrocytes (Conti et al., 2005; Glaser et al., 2007). Furthermore, they express eCB signaling enzymes and respond to them, as will be discussed below. We therefore concluded that the Cor-1 cells are a suitable *in vitro* model to investigate the role of eCBs in neural progenitor cells. As with other *in vitro* models, this imposes certain limitations and any findings need to be validated *in vivo,* which has been done successfully for certain aspects of our Cor-1 cells work in the context of proliferation and migration (Goncalves et al., 2008; Oudin et al., 2011b).

NSC lines including the Cor-1 cells appear to express the DAGL enzymes at a higher level compared to other cell lines (Goncalves et al., 2008) and moreover DAGLα mRNA has been established by real-time PCR (PhD Thesis Dr Debbie Walker, Doherty lab). CB1 as well as CB2 receptor expression has been shown on mRNA as well as protein level in NSC lines. FAAH and MAGL can both break down 2-AG *in vitro* and therefore are a key part of the eCB signaling system. While FAAH primarily breaks down anandamide *in vivo*, MAGL has been shown to mainly degrade 2-AG alongside other non-eCB monoacylglycerols (reviewed in De Petrocellis et al., 2004). NSCs therefore express various components of the eCB system and the expression of CB1 receptor, FAAH, and DAGLα in Cor-1 cells has been verified in Figure 3.2.

DAGLα is expressed by proliferating cells in the SVZ (Goncalves et al., 2008) while CB1 and CB2 receptors are expressed in progenitor cells of the hippocampus (Aguado et al., 2006; Palazuelos et al., 2008). Moreover, DAGL and CB2 receptor antagonists can inhibit the proliferation of progenitor cells in mice and CB2 receptor agonism stimulates the proliferation of progenitor cells *in vivo*, which is an effect

more pronounced in older animals. DAGLα, CB1, and CB2 receptors are expressed by nestin positive cells in neurospheres *in vitro* (Molina-Holgado et al., 2007). Upon calcium-ionophore stimulations, neurospheres release 2-AG as well as lower amounts of anandamide (Aguado et al., 2005). The proliferation of nestin-positive cells within neurospheres is increased when the CB1 receptor is stimulated either by a synthetic agonist or by preventing eCB breakdown which indicates that not only are eCBs being made by these cells but they are also able to react to eCB stimulation (Aguado et al., 2005). The proliferation of cultured NSCs can be inhibited with DAGL as well as with CB2 receptor antagonists (Goncalves et al., 2008). These *in vitro* and *in vivo* results combined make a convincing case for neural progenitors being responsive to eCB signaling and that their proliferation is influenced by them. The novel DAGL inhibitor OMDM-188 has been explored in this context and was shown to significantly inhibit Cor-1 cell proliferation (Figure 3.3).

Next we wanted to investigate the role of the eCB system on differentiation. Using a neuronal differentiation protocol adapted from Conti, Pollard et al. 2005, the Cor-1 cells were differentiated and characterised. At least a subpopulation of cells remains proliferative and cells display an elongated, more branched morphology after 4 to 7d of proliferation (Figure 3.4). A mixed lineage population of cells, with some cells expressing the Tuj-1 neuronal marker, and other cells expressing the astrocytic marker GFAP (Figure 3.12) was generated, while other cells appeared to belong to yet other categories. The inhibition or activation of CB1 and/or CB2 receptors had no effect on the percentage of cells adopting a neuronal phenotype as assessed by Tuj-1 staining (Figure 3.5-3.6). Thus we conclude that the eCB system does not play a key role in the differentiation of Cor-1 cells along the neuronal lineage in Cor-1 cells. This is in line with *in vivo* findings showing that the number of cells committing to neuronal differentiation is not changed significantly in CB1 receptor or FAAH KO mice (Aguado et al., 2006). In migratory neuroblasts travelling though the RMS however the eCB system has been shown to be involved in migratory processes such as nucleokinesis and cell branching displaying another role for the eCB system in neuronal differentiation (Oudin et al., 2011b).

In the hippocampus the CB1 receptor is expressed by astrocytes and is involved in the communication between astrocytes and neurons (Coiret et al., 2012). This demonstrates a role for the eCB system in astrocytes and eCB signaling has been demonstrated to induce astroglial differentiation of neural progenitor cells *in vitro*. Moreover opposing astroglial differentiations patterns were observed for CB1 receptor KO and FAAH KO mice. While astroglial differentiation was decreased in CB1 receptor KO mice it was increased in FAAH KO mice (Aguado et al., 2006). We therefore were interested in the role of eCB signaling in astrocyte differentiation of Cor-1 cells. Using an astrocytic differentiation protocol, Cor-1 cells were differentiated to cells displaying a star shaped, astrocytic morphology and expressing GFAP (Figure 3.7). CB1 and/or CB2 receptor antagonists had no effect on the differentiation of Cor-1 cells to astrocytes (Figure 3.8). This indicates that the eCB system does not play a role in astrocytic differentiation of Cor-1 cells. Therefore the role of eCB signaling in astrocyte differentiation might be context dependent. A role for the eCB system in oligodendrocyte differentiation has been reported (Gomez et al., 2010) and more recently the PI3 kinase/ Akt and mTOR pathway have been shown to be part of the signaling cascade in this context (Gomez et al., 2011).

Cor-1 cell lines overexpressing DAGLα and DAGLβ were established (Dr Philipp Sütterlin; Figures 3.9-3.10). For further studies on the enzymatic activity of DAGLα/β in these cell lines see Chapter 6, where a surrogate substrate assay was used to confirm the increased DAGL dependent enzymatic activity of these cell lines. While DAGLα-V5 staining is observed throughout the whole cell surface, DAGLβ staining is very punctuated. This also was observed in other clonal Cor-1 cell lines overexpressing the DAGLβ construct (PhD thesis Philipp Sütterlin). While it is possible that DAGLβ is not localized to the cell surface in general, another possibility would be that DAGLβ needs another enzyme such as DAGLα to be correctly localized. To test this hypothesis it would be necessary to double transfect the cells with DAGLβ as well as with DAGLα for example and analyse whether this leads to cell surface localization. Compared to DAGLα, little is known about the expression pattern of DAGLβ. In my opinion this reflects the absence - to

my knowledge - of a suitable DAGLβ antibody for immunostaining as well as the absence of selective DAGLα/β inhibitors. Observations in the DAGLβ KO mice (Gao et al., 2010) should help to address this in the future and will help to distinguish between DAGLα and β functions. Moreover other proteins might form complexes with DAGLα and/or DAGLβ, which might aid them in their localisation as well as functionality. To date only two proteins are known to interact with DAGLα. These proteins are Homer -1b and Homer-2 (Jung et al., 2007). It would be very interesting to expand our knowledge about other potential interaction partners. Several proteins have been investigated in this context by our lab (unpublished data), but further evidence is needed to establish the possible relationship between the potential interaction partners and the DAGLs. The DAGLα-V5 overexpressing cells were taken forward for further characterization and they do not display a significantly different morphology compared to the parental Cor-1 cells (3.11). This indicates that DAGLα does not have an influence on Cor-1 cell shape.

Next we wanted to establish the expression of DAGLα in neuronally differentiated Cor-1 cells. To address this question, DAGLα expression was examined by western blotting over a 4d period of differentiation toward a neuronal phenotype. A dramatic reduction in expression was seen, and this was recapitulated with an epitope tagged version of DAGLα expressed under the control of a CMV-promoter (Figure 3.12). Immunocytochemistry confirmed a rapid reduction of endogenous and transgenic DAGLα in Tuj-1 positive cells (Figure 3.13), opening up the question of how DAGLα expression is reduced so quickly in these cells. The rapid reduction of DAGLα in Tuj-1 positive interneurons is somewhat surprising given the proposed role of DAGLα for neurite outgrowth during development. Some studies suggest that DAGLα is not expressed by GABAergic interneurons but by pyramidal interneurons neurons during development (Mulder et al., 2008). Therefore it would be interesting to explore different neuronal differentiation protocols to create other types of interneurons and establish DAGLα expression in that context.

Given the change in DAGLα during neuronal differentiation we wanted to establish the DAGLα expression levels during astrocytic differentiation of Cor-1 cells. Endogenous DAGLα expression was not changed significantly upon differentiation

of Cor-1 cells to astrocytes, however transgenic DAGLα-V5 expression increased quite dramatically (Figure 3.14). The reasons for this are not as clear, but one obvious possibility is a higher activity of the CMV promoter in astrocytes as compared to undifferentiated Cor-1 cells.

As mentioned above, DAGLα expression is significantly reduced in interneurons. In the next chapter we therefore wanted to explore the possibility of DAGLα regulation on the protein level.

CHAPTER IV: Results 2

Investigation of DAGLα degradation

4.1. Introduction

Gene expression is usually controlled by transcriptional regulation, but post translational modifications allow an alternative regulation mechanism. Very little information is available on the regulation of DAGL expression at the transcriptional or protein level. Based on the observation that DAGLα expression is down-regulated upon neuronal differentiation (see sections above) and our findings indicate that this is unlikely to be solely due to transcriptional regulation, we decided to investigate the post-transcriptional regulation of the DAGLs.

Several proteins in neurons have been discovered to be targeted for degradation via a motif called a destruction box (d-box), which marks them for degradation via the ubiquitin-proteasome pathway (Hershko and Ciechanover, 1998; Hershko, 2005). A putative d-box has been identified in the DAGLα sequence and we wanted to investigate if this targets DAGLα for degradation. Only 10-20% of the d-box sequence is conserved with the core sequence being RxxLxxxxN/D/E. The single amino acid code of this sequence contains an invariable arginine and lysine, and it has been shown, that mutation of the invariant arginine prevents the degradation of cyclins (Glotzer et al, 1991). The d-box marks the enzyme as a substrate for the ubiquitin ligase anaphase-promoting complex (APC) and substrate recruitment often requires a co-activator such as Cdh1 and Cdc20 (reviewed in Kim and Bonni, 2007). After the protein has been conjugated by one or more ubiquitin molecules, which is a highly conserved small protein, they are destined to be degraded by the 26S proteasome. The ubiquitin proteasome pathway is the most significant degradation pathway other than the lysosome pathway. This degradation pathway carries out the selective degradation of many proteins in eukaryotic cells (Hershko and Ciechanover, 1998; Hershko, 2005; Varshavsky, 2012).

4.2. Establishing a Cor-1 cell line expressing DAGLα-V5 with a silenced, putative d-box

A putative d-box motif was discovered within the sequence of DAGLα via a bioinformatic approach (personal communications, Dr Gareth Williams). We created a plasmid containing the DAGLα sequence expressed under the CMV promoter with the invariable arginine and lysine changed for alanines. This should prevent d-box dependent degradation. Cor-1 cells can be efficiently transfected using the AMAXA nucleofection technology and were nucleofected with the mutated d-box plasmid. Stable Cor-1 cell lines expressing DAGLα containing the putative d-box mutations were selected in G418 containing media (see methods for details) and the most suitable cell line was selected based on western blot analysis and immunocytochemistry.

Different clonal colonies were grown up and their V5-DAGLα-m-dbox expression levels were established (Figure 4.1). The two clones showing the highest expression levels in preliminary experiments were Mα8 and Mα11. They were grown up in selection media and their protein was extracted and used for western blot analysis. An anti-V5 antibody was used to compare expression levels of V5α7, Mα8, and Mα11 (Figure 4.1 B). An anti-actin antibody was used as a loading control and Mα8 displays a good expression level. It is lower than the expression level seen in V5α7 lysates, but considerably higher than the expression levels in Mα11 cells or the non selective cell line. Immunocytochemistry was carried out to confirm a uniform expression throughout the Mα8 cell line and 100% of the cells are positive for DAGLα-V5-m-dbox (Figure 4.1 A). This was deemed to be a suitable cell line to carry our further experiments.

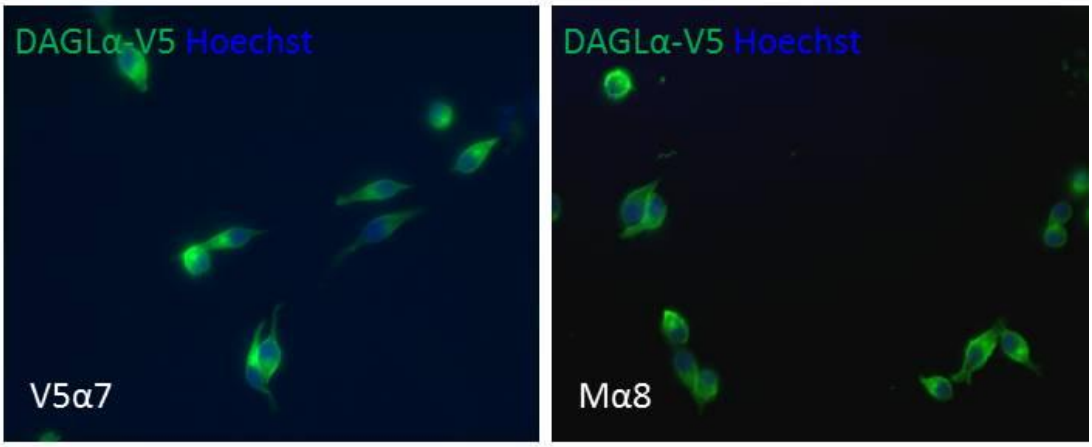
4.3. Neuronal differentiation of the Mα8 cell line

The Mα8 cell line was used to determine if the mutation within the d-box had any effect on the loss of DAGLα expression previously seen during neuronal differentiation. The expression levels of the DAGLα-V5-m-dbox in Mα8 cells at 0-4d of neuronal differentiation was monitored. Similar to the situation seen with endogenous DAGLα, and the V5-tagged wt DAGLα, the expression levels of DAGLα-V5-m-dbox drops rapidly upon neuronal differentiation (Figure 4.2).

A

D-box motif: R-X- X- L-X- X- X-N/D/E
DAGLα sequence: ^{892}R-G-E-L-A-L-H-N-G
mutated DAGLα sequence: ^{892}A-G-E-A-A-L-H-N-G

B



C

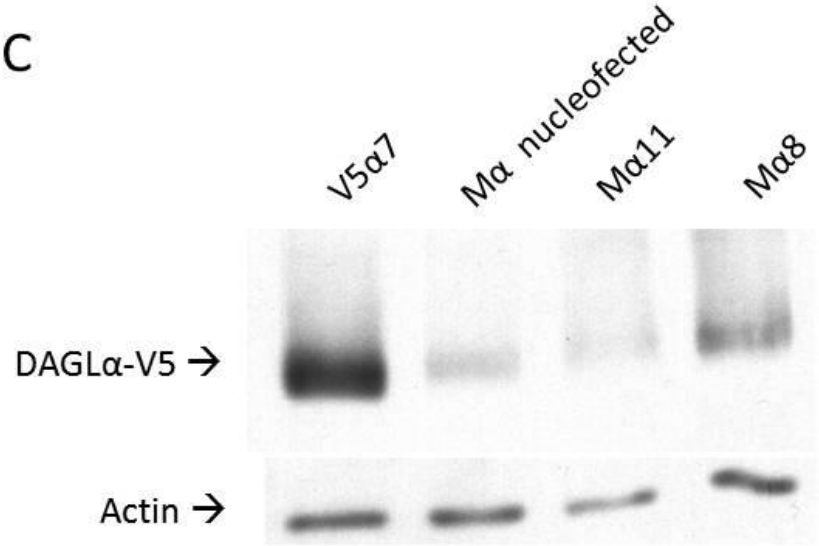
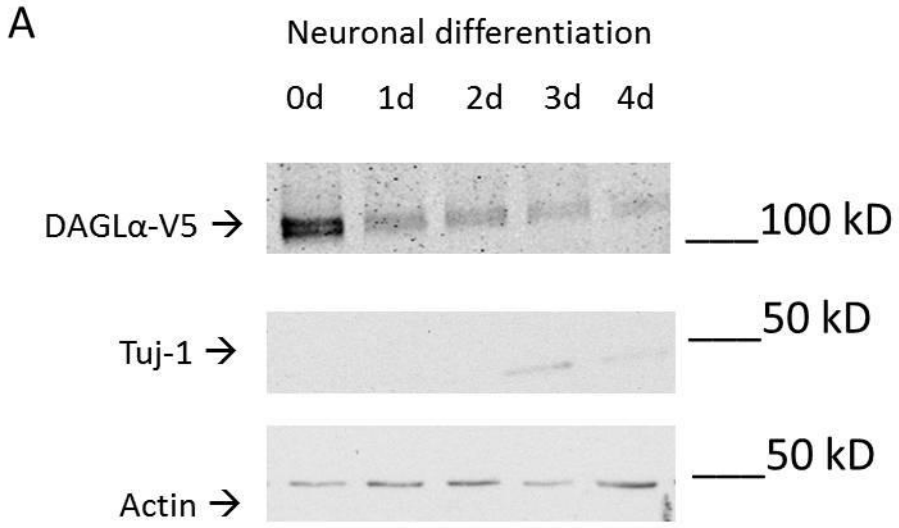


Figure 4.1: Creation of a Cor-1 cell line expressing a DAGLα-V5 construct with a mutated, putative d-box

The putative d-box sequence in human DAGLα is presented and the conserved amino acids are highlighted in blue. Site directed mutagenesis was used to induce an amino acid change to alanine for the conserved amino acids (A). Cor-1 cells were either nucleofected with a DAGLα-V5 (V5α7) construct or with a DAGLα-V5 construct with a mutated d-box (Mα8). Clonal cell lines were created from those cells. Cells were stained with a anti-V5 antibody for the overexpressed DAGLα-V5. Very similar expression patterns were observed when comparing V5α7 and Mα8 (B). Western blot analysis was used to compare the level of protein expression. Actin was used as a loading control.


A
Neuronal differentiation
0d
1d
2d
3d
4d
DAGLα-V5 →
100 kD
Tuj-1 →
50 kD
Actin →
50 kD

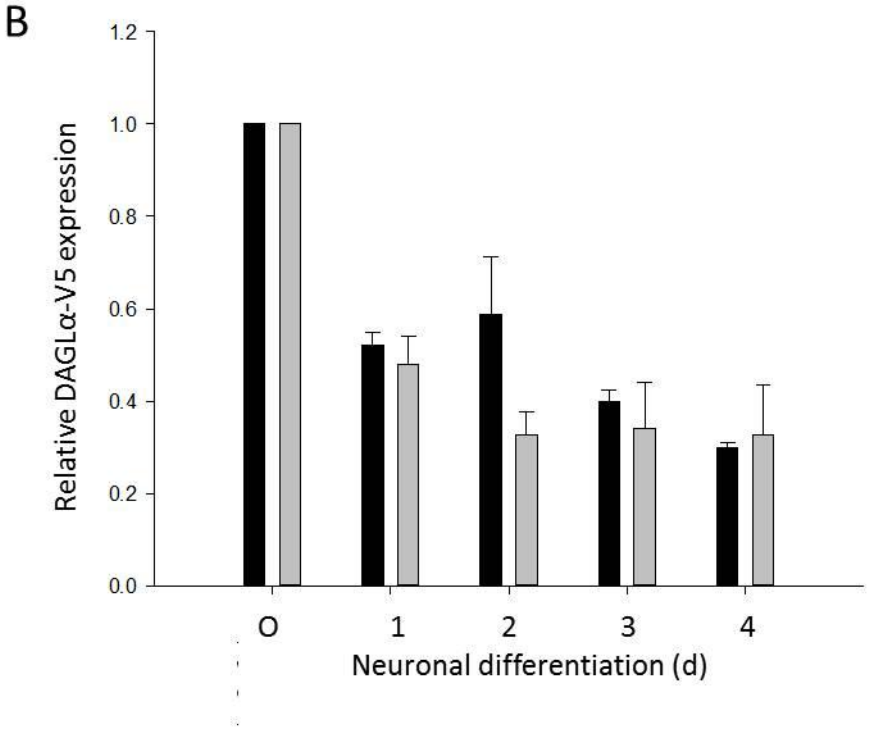

B
Relative DAGLα-V5 expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O
1
2
3
4
Neuronal differentiation (d)

Figure 4.2: Comparison between transgenic DAGLα expression in V5α7 cells and Mα8 cells

Mα8 and V5α7 cells were seeded at 500,000 cells/well into 6-well plates coated with laminin and poly-ornithine and left to attach overnight in expansion media. Afterwards, the media was changed to neuronal differentiation media. Cells were differentiated for 0-4d before protein extraction. Western blot analysis was performed. An anti-V5 antibody was used to analyse the transgenic expression of DAGLα-V5 and a representative picture of Mα8 cells is presented. An anti-Tuj-1 antibody blotting confirms neuronal differentiation and an anti-actin antibody was used as loading control (A). The band intensity was quantified using ImageJ analysis and is displayed as a ratio of DAGLα-V5/Actin. The data was normalised to 0d of differentiation and the mean of three different experiment is displayed ± SEM. No significant difference was detected between the cell lines (student t-test) (B).

The relative expression levels normalised to 0d of differentiation relative to actin is presented in Figure 4.2 B. The transgenic DAGL expression was compared between V5α7 and Mα8 cells and no fundamental difference was detected. This indicates that the mutation within the putative d-box in the transgenic DAGLα-V5 enzyme does not affect the stability of the enzyme, with the same rate of loss seen upon neuronal differentiation.

The Mα8 cells were seeded onto laminin and poly-ornithine coated glass cover slips and differentiated using a neuronal differentiation protocol as described earlier for 7d. Double labelling the cells with an anti-Tuj-1 and anti-V5 antibody (Figure 4.3) confirmed rapid reduction of the d-box mutated DAGLα from the Tuj-1 positive cells, indicating that the d-box mutation has no effect on down-regulation of DAGLα during neuronal differentiation.

4.4. DAGLα can be ubiquitinated

Our results point towards a d-box independent mechanism for DAGLα down-regulation upon neuronal differentiation. The ubiquitin-proteasome pathway is one of the most significant protein specific recycle mechanisms in mammalian cells. We therefore investigated the possibility that DAGLα might be ubiquitinated independently from a d-box motif. IPs from the V5α7 cells using an anti-V5 antibody were carried out and the samples were used for western blot analysis (Figure 4.4). A generic anti-ubiquitin antibody was used to detect all ubiquitinated proteins within the lysate and multiple bands are detected in the lysate lane. The most pronounced band however is found at 120 kDa, which is the molecular weight of DAGLα. Using an anti-V5 antibody this band was confirmed to be DAGLα-V5.

We next wanted to investigate the effect of neuronal differentiation on DAGLα ubiquitination. V5α7 cells were differentiated for 4d using a neuronal differentiation protocol. These cells as well as undifferentiated control cells were used for IPs using an anti-V5 antibody to pull down DAGLα-V5. IPs without antibody were carried out as a negative control and these samples as well as lysate samples were used for western blot analysis (Figure 4.5).

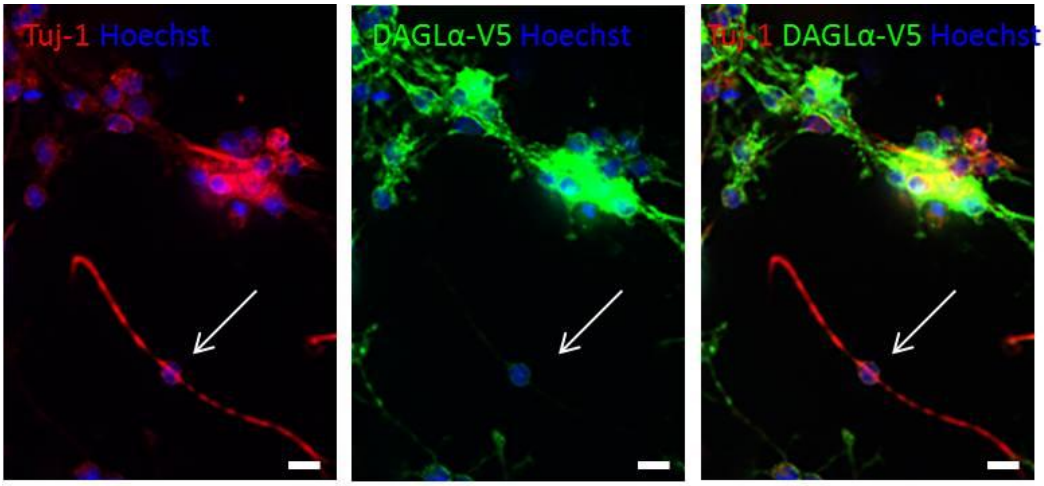


Figure 4.3: DAGLα-V5 down-regulation during neuronal differentiation of Mα8 cells

Mα8 cells were differentiated with a neuronal differentiation protocol for 7d. 10,000 cells/well were seeded onto laminin and poly-ornithine coated glass cover slips in 4-well plates and cells were allowed to attach over night in expansion media. Then the media was changed to neuronal differentiation media containing B27, 5ng/ml FGF-2 and no EGF. Half the media was exchanged after 4d for fresh neuronal differentiation media. The cells were fixed after 7d using PFA and stained with an anti Tuj-1 and anti-V5 antibody. The nucleus was labelled with Hoechst. Scale bars: 10µM

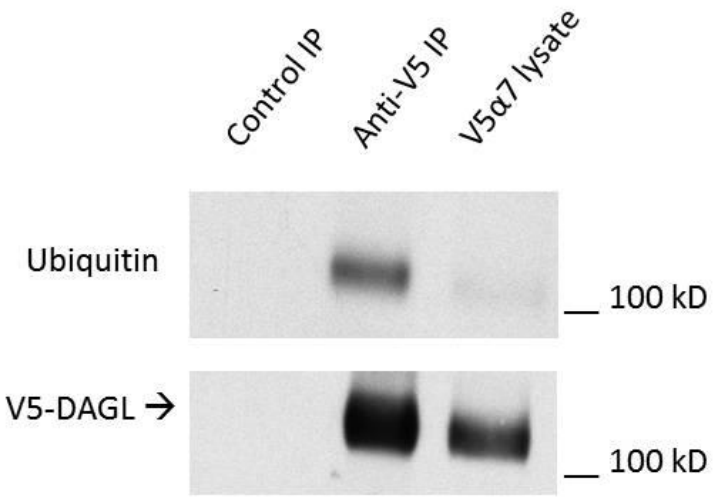


Figure 4.4: DAGLα-V5 is ubiquitinated in V5α7 cells

V5α7 cells were grown in normal growth media and the protein was extracted from them. DAGLα-V5 was immunoprecipitated using an anti-V5 antibody. As a negative control a no antibody immunoprecipitation (IP) was run as well. These samples as well as a lysate sample were used for western blot analysis. An anti-ubiquitin antibody was used to detect all ubiquitinated proteins. Next, an anti-V5 antibody was used to visualize DAGLα-V5.

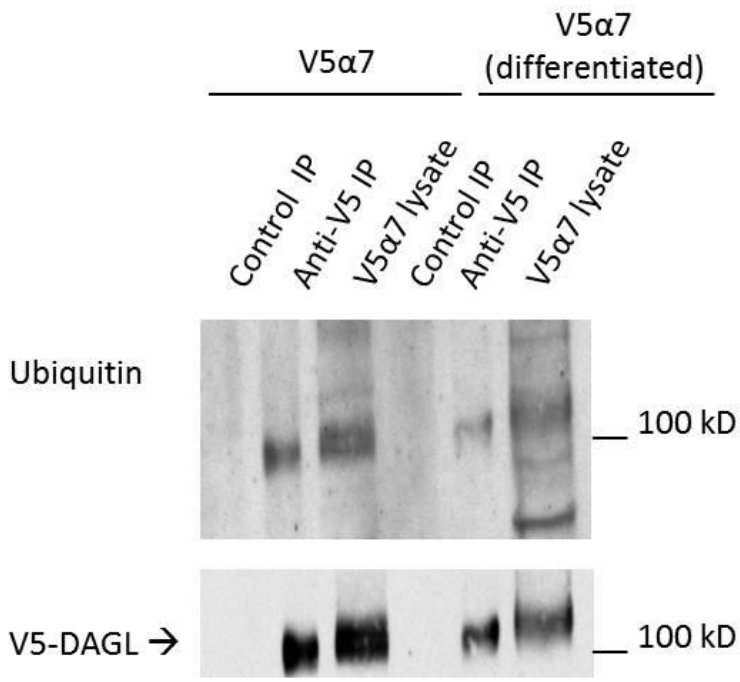


Figure 4.5: DAGLα-V5 can be ubiquitinated in undifferentiated and differentiated cells

V5α7 cells were either grown in normal expansion media or neuronal differentiation media for 4d, changing half the media for fresh differentiation media on day 2. Lysates were used for immunoprecipitation (IP) using an anti-V5 antibody to immunoprecipitate DAGLα-V5, or no antibody as a negative control. These samples as well as a lysate sample were used for western blot analysis. An anti-ubiquitin antibody was used to detect all ubiquitinated proteins. Next, an anti-V5 antibody was used to visualize DAGLα-V5.

An anti-ubiquitin antibody was used to detect all ubiquitinated protein and multiple bands were detected in the lysate band. A band at 120 kDa is detected in the IP lanes, but not the negative IP bands for the differentiated as well as the undifferentiated V5α7 cells. The membranes were stripped and an anti-V5 antibody was used to confirm the 120 kDa band to be DAGLα. The ubiquitin antibody clearly labelled the immunoprecipitated V5-DAGLα from both the control and differentiated cells (top panel in Figure 4.5), with the reduced level of labelling seen in the differentiated cells accountable for the reduced level of V5-DAGLα in the immunoprecipitate (bottom panel Figure 4.5). This shows that DAGLα-V5 can be ubiquitinated in the differentiated cultures with no obvious evidence for any major change in this relative to the control cultures.

4.5. Inhibition of proteasomal degradation

Next we further wanted to establish, if DAGLα ubiquitination leads to its degradation via the proteasome. Lactacystin is a selective inhibitor of the proteasome complex (Omura et al., 1991). If ubiquitinylation of DAGLα-V5 leads to its degradation, the use of Lactacystin should prevent its degradation and increase its' expression levels.

Cor-1 cells were treated with 0.5µM Lactacystin for 24h and pictures were taken every 2h with the IncuCyte platform (Figure 4.6) and the cell proliferation and morphology was observed. Cor-1 proliferation was inhibited and cells processes appeared shorter. Overall the cells appeared more rounded, but about 50% of the cells still had processes after 6h of Lactacystin treatment indicating that they were alive (Figure 4.6 A).

To investigate the effect of proteasome inhibition on DAGLα protein levels, we treated Cor-1 cells with 0.5µM Lactacystin for 0, 2, 4, or 6h before extracting their protein for western blot analysis. A DAGLα antibody that recognises the endogenous enzyme was used to quantitate expression, and as expected it recognised a single a band 120kDa (Figure 4.7). Actin was used as a loading control. The intensity of the band was measured using the inbuilt Odyssey software and expression levels relative to actin established.

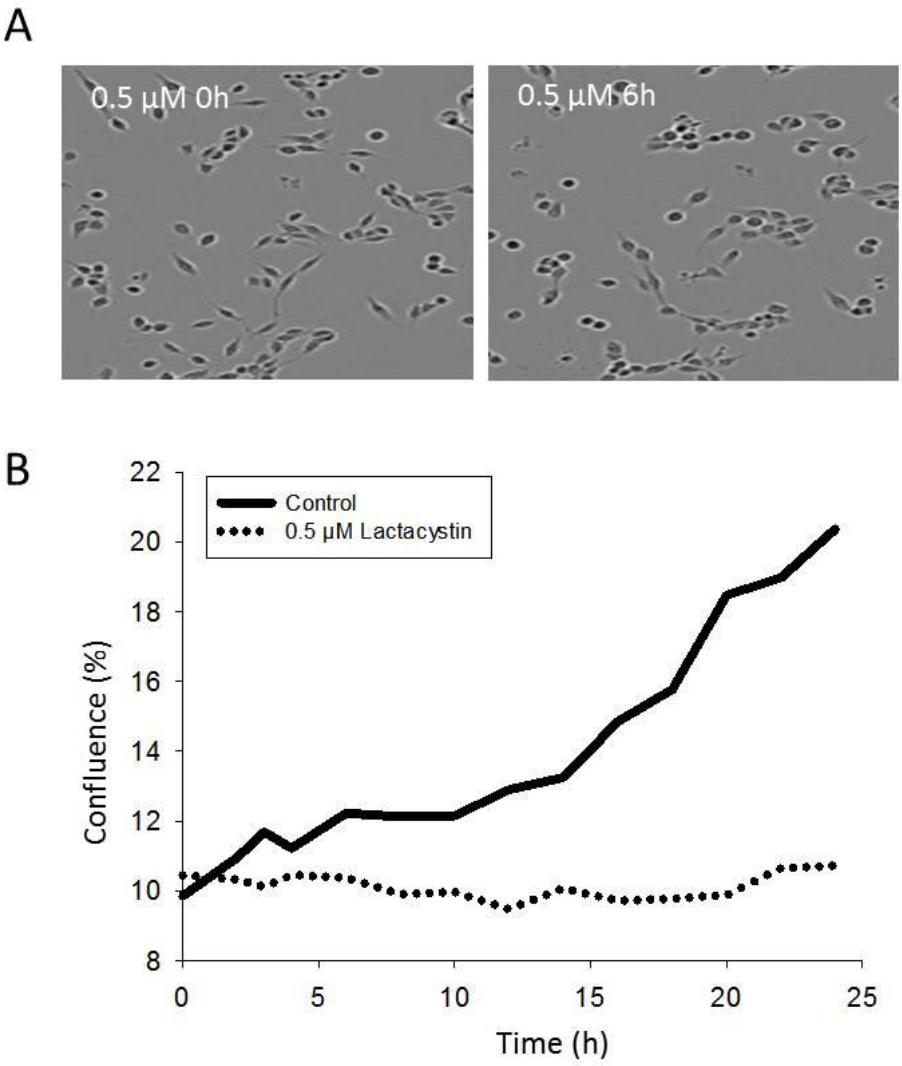


Figure 4.6: Cor-1 proliferation is reduced by lactacystin treatment

Cor-1 cells were seeded at a density of 8,000 cells/well and left overnight to attach. The cells were then treated with 0-0.5µM lactacystin, which is a selective proteasome inhibitor. Phase-contrast pictures were taken every 2h for 24h using IncuCyte imaging. Representative pictures taken at 0 and 6h of 0.5µM lactacystin treatment are presented in A, while cell confluence is presented in B and shown as the mean from 6 wells.

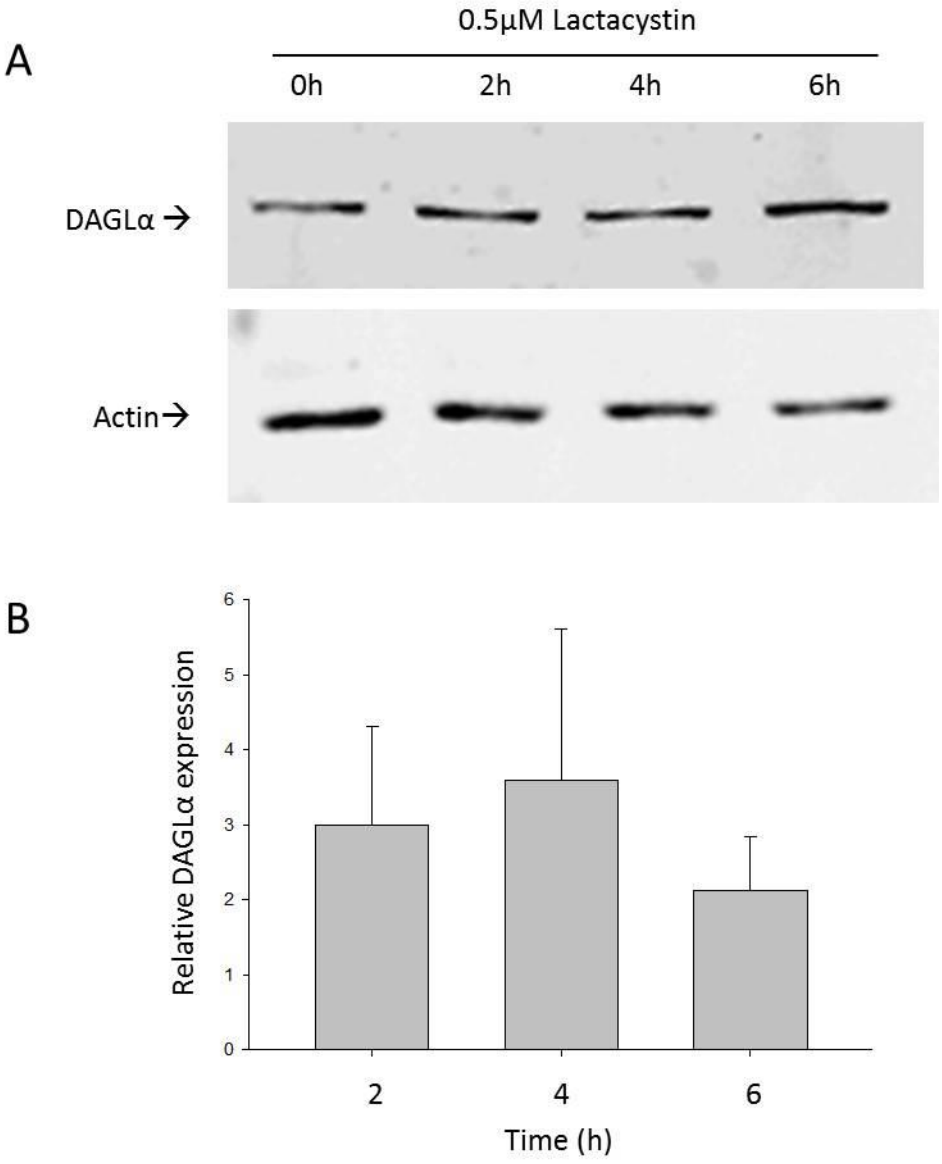


Figure 4.7: DAGLα expression following 0, 2, 4 and 6h of lactacystin treatment

Cor-1 cells were treated with lactacystin for 0, 2, 4 or 6h before proteins were extracted. Samples were used for western blot analysis and representative images for DAGLα and actin can be seen in A. The band intensity was measured using the Odyssey software and DAGLα expression relative to total protein (actin) is presented in B normalised to the 0h control. The mean of three independent experiments ± SEM is shown. No significant difference was observed (anova and student t-test).

The expression was normalised to the control, and the mean of three independent experiments ± SEM is presented in Figure 4.7 B. Although there was a trend suggesting a rapid increase, this was not significant due to the high variability between the three experiments.

4.6. Conclusions and discussion

Following our observation that Cor-1 cells differentiated to interneurons display a rapid decrease in DAGLα expression, we wanted to explore the mechanisms that regulate DAGLα degradation during differentiation. Very little is known about post translational modifications of the DAGLs as well as other regulatory mechanisms. To investigate DAGL degradation a DAGLα-V5 overexpressing Cor-1 cell line with mutations in key residues in a putative d-box was established and called Mα8 (Figure 4.1). The cell line appears to be clonal as demonstrated by expression of the mutated construct in all cells. Compared to the cell line V5α7 expressing wt DAGLα-V5, expression levels in the Mα8 cells are slightly lower, but nonetheless at reasonable levels that are readily detectable.

The effect of neuronal differentiation on transfected DAGLα levels in Mα8 cells was investigated. V5α7 cells and Mα8 cells were differentiated for 0-4d. In both cases a rapid drop in overall transgenic DAGLα-V5 expression was seen (Figure 4.2) as well as a rapid reduction in DAGLα-V5 expression in Tuj-1 positive cells (Figure 4.3). Together these results show that in the context of neuronal differentiation of Cor-1 cells, the putative d-box has no effect on its degradation.

The DAGLα-V5 plasmid with the d-box mutation plasmid was used for experiments using the hippocampi from E18 rats. The hippocampi were dissected and nucleofected with 5µg of either the DAGLα-V5 plasmid or the DAGLα-V5 plasmid with the mutated d-box. The cells were plated onto poly-lysine and laminin coated glass coverslips and grown for 2d under suitable conditions after which they were fixed and used for immunocytochemistry. The cells were co-stained with Tuj-1 and V5 and in both cases, the DAGLα-V5 or DAGLα-V5 with a mutated d-box, V5 is present throughout the whole cell. Co-stainings with V5 and Tuj-1 showing co-localisation of V5 and Tuj-1 show that the d-box has no effect on DAGLα localization

in immature hippocampal neurons (Dr Madeleine Oudin, PhD thesis). Following the same experimental set up, cells were grown for 14d before being fixed and used for immunocytochemistry. The cells were stained for microtubule-associated protein 2 (MAP2) which is enriched in neuronal dendrites and it was used for a co-staining with V5 showing that DAGLα-V5 is largely expressed in the dendrites. This was not changed by the d box mutation indicating that the putative d-box is not involved in the exclusion of DAGLα from the axons in mature hippocampal neurons (Dr Madeleine Oudin, PhD thesis).

To further investigate DAGLα degradation pathways we next wanted to investigate the ubiquitin proteasome pathway. Protein degradation via both the proteasome as well as lysosome pathway can be directed via ubiquitination and are regarded as the most important degradation pathways in mammalian cells (Hershko and Ciechanover, 1998; Piper and Luzio, 2007). As a first step we therefore investigated if DAGLα can be ubiquitinated. IPs of DAGLα-V5 with an anti-V5 antibody revealed that the enzyme can be ubiquitinated (Figure 4.4). This process was not obviously altered by neuronal differentiation (Figure 4.5). Due to the very low levels of endogenous DAGL seen in differentiated culture, we were unable to verify this data for the endogenous DAGLα enzyme. Since this observation was made in a DAGLα-V5 overexpressing cells line it remains an open question if this form of post translational modification also occurs when the enzyme is expressed at a "normal" level.

To further investigate the possibility of DAGLα being degraded via the ubiquitin proteasome pathway, we treated Cor-1 cells with 0.5μM lacatcystin, which resulted in inhibition of cell proliferation. Inhibition of cell proliferation also has been observed in other studies of neuroblastoma cells such as Neuro-2a alongside inhibition of neurite outgrowth (Omura et al., 1991; Fenteany et al., 1994). Higher concentrations lead to cell death within a short period of time (data not shown), which demonstrates the importance of protein recycling for cell health and cells seem to be more sensitive to Lactacystin treatment in comparison with for example epithelial cells (Fenteany et al., 1994). If DAGLα is degraded via the proteasome pathway its' inhibition should lead to an increase in DAGLα protein levels. We

therefore treated Cor-1 cells with 0.5µM Lactacystin between 0-6h and found no fundamental change in DAGLα expression levels in the observed time period. It has to be mentioned, that a substantial variation was observed between experiments. Therefore further experiments are needed to establish the role of the proteasome complex for DAGLα degradation.

To further investigate the ubiquitination status of DAGLα, an E1- ligase inhibitor could be used. While cells have multiple E2 and E3 type enzymes, they typically only have one E1 ligase. Such type of inhibitor has been described and would theoretically inhibit all ubiquitination events in the cell (Yang et al., 2007). Alternatively other inhibitors blocking protein ubiquitin related protein degradation like Bortezomib could be used (Adams and Kauffman, 2004). In order to narrow down the exact ubiquitination process, pseudosubstrate inhibitors like Emi1 could be used to inhibit the APC complex and other parts of the ubiquitination process (Reimann et al., 2001; Straiker et al., 2009). Ubiquitin also has been shown to potentially play a role in lysosomal degradation. In the context of neurodegenerative diseases ubiquitin conjugated protein clusters have been found in presumably lysosomal complexes (Blommaart et al., 1997). Lysosome specific inhibitors are also available and could be used to explore this line of degradation (Qin et al., 2003).

CHAPTER V: Results 3

Upstream and downstream cascade of DAGLα/eCB signaling in Cor-1 cells

5.1. Introduction

Optimal growth of Cor-1, and other NSC lines, is dependent on a mixture of nutrients as well as growth factors such as EGF and FGF-2 (Conti et al., 2005; Sun et al., 2008). Furthermore, these growth factors play an important role in neurogenesis *in vivo* (Tropepe et al., 1997; Zhang et al., 2004) and play a crucial role in the generation of NSC cultures (Pollard et al., 2006). Whereas EGF is crucial for maintaining cell survival and proliferation, absence of FGF-2 signaling only decreases cell proliferation (Pollard et al., 2006). The EGFR and FGFR are both receptor tyrosine kinases (RTKs) and are well placed to regulate DAGLα/β activity through PLCy. Furthermore, the FGFR has been demonstrated to be upstream of a DAGL-eCB response during development and to mediate neurite outgrowth as well as path finding (Williams et al., 2003). Another substance which stood out from the nutrient mix is insulin. In order to adapt to environmental changes, stem cells have to be able to detect and react to metabolic fluctuations (Speder et al., 2011) and insulin signaling was recently linked to brain cell progenitor proliferation (Hodge et al., 2004; Popken et al., 2004; Lehtinen et al., 2011).

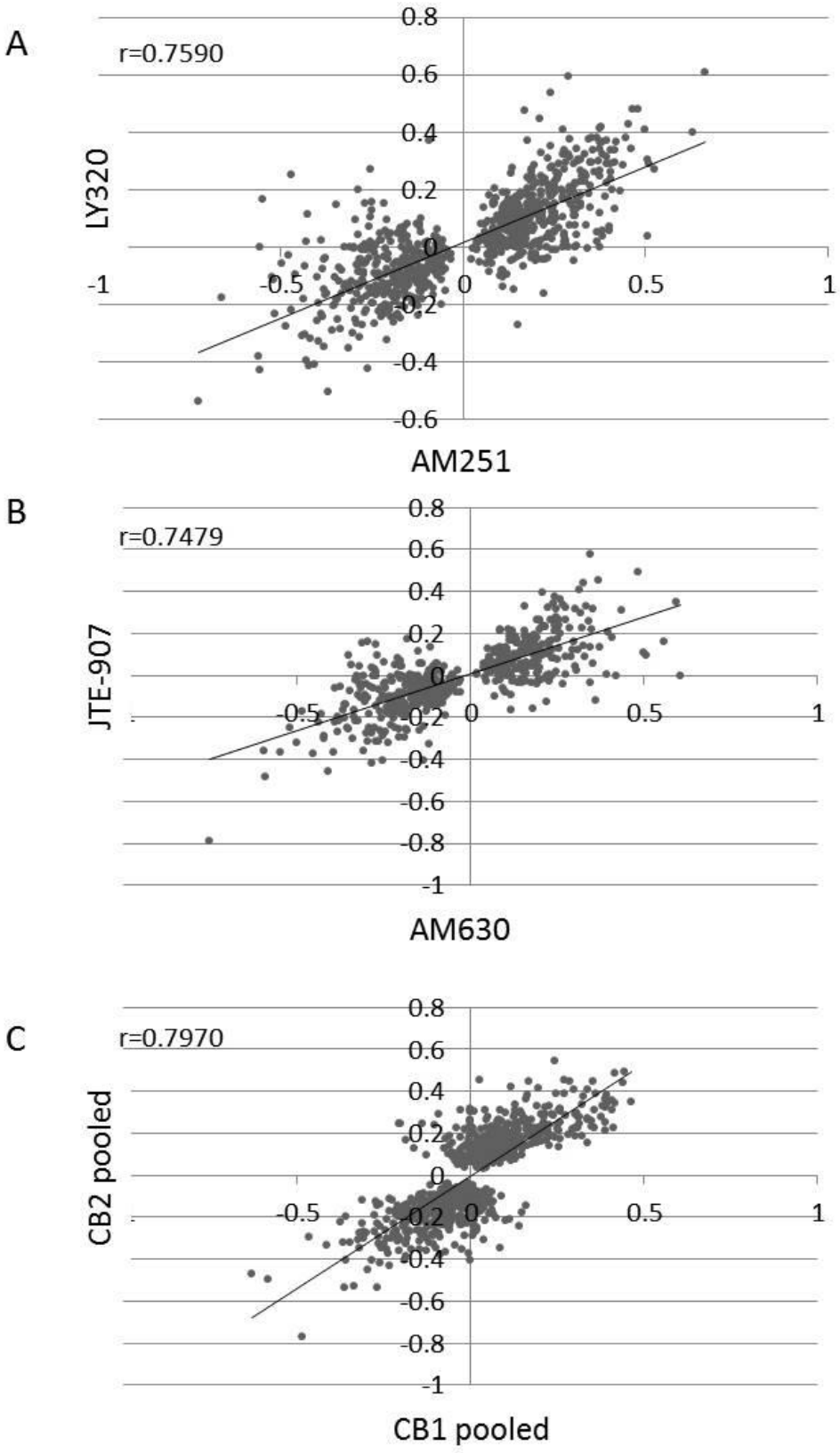
Cor-1 and other NSC lines have an endogenous eCB tone that drives proliferation, with nothing known about how this tone is generated. Given the established cross-talk between receptor tyrosine kinases and eCB signaling, we wanted to determine if EGF and FGF-2, or insulin might be responsible for the eCB tone in Cor-1 cells.

5.2. What drives eCB signaling?

5.2.1. Micro array analysis comparing eCB, EGFR and FGFR signaling

One way to determine the relationship between EGF, FGF, and eCB signaling is to identify the transcripts regulated by each receptor and see if the transcriptional responses are related. We used a microarray approach to analyse the transcriptional response of eCB as well as EGFR and FGFR signaling in Cor-1 cells and compared the responses. Two structurally independent CB1 (1µm AM251, 1µM LY320135) and CB2 (1µM AM630 and 1µM JTE907) receptor antagonists were used. Furthermore the EGFR antagonists AG1478 and PD168393 (both 100nM) and FGFR antagonist PD173074 (500nM) were used. Drugs were dissolved in 1000X DMS0 and compared with a 0.1% DMSO treated control. Cor-1 cells were grown on T75 tissue culture flasks and treated for 4h with the respective antagonists. The mRNA was extracted in RLT buffer (Qiagen RNeasy kit) and frozen on dry ice. Samples were collected on three independent and consecutive days for all treatment groups as well as the control. The micro array analysis was carried out by Wyeth Research (now Pfizer) collaborators in Boston. The data of three different days was averaged and compared to the control.

To analyse the microarray date, values below 20 were excluded and treatment response was defined as $2\frac{\mathrm{t-c}}{t+c}$ with t being the average treatment value and c being the average control value. Statistical significance was established using the student t-test. Significant responders to AM251 treatment were compared with all responders to LY320 treatment. Pearson's correlation coefficient r created values between -1 and 1. The correlation coefficient of r=0.7590 between AM251 and LY320 is very high (Figure 5.1 A). A similar result was obtained when comparing the significant AM630 responders with the total JTE970 (Figure 5.1 B). Based on their high correlation, the CB1 receptor responders obtained with the AM251 and LY320 and CB2 receptor responders to AM630 and JTE907 were pooled and compared with each other (Figure 5.1 C). The correlation coefficient of r=0.7970 confirms a strong correlation between CB1 and CB2 receptor signaling. This demonstrates that CB1 and CB2 receptors have a similar transcriptional response in Cor-1 cells. Therefore they were pooled before comparing them with EGFR and FGFR inhibitors.


A
r=0.7590
LY320
AM251
B
r=0.7479
JTE-907
AM630
C
r=0.7970
CB2 pooled
CB1 pooled

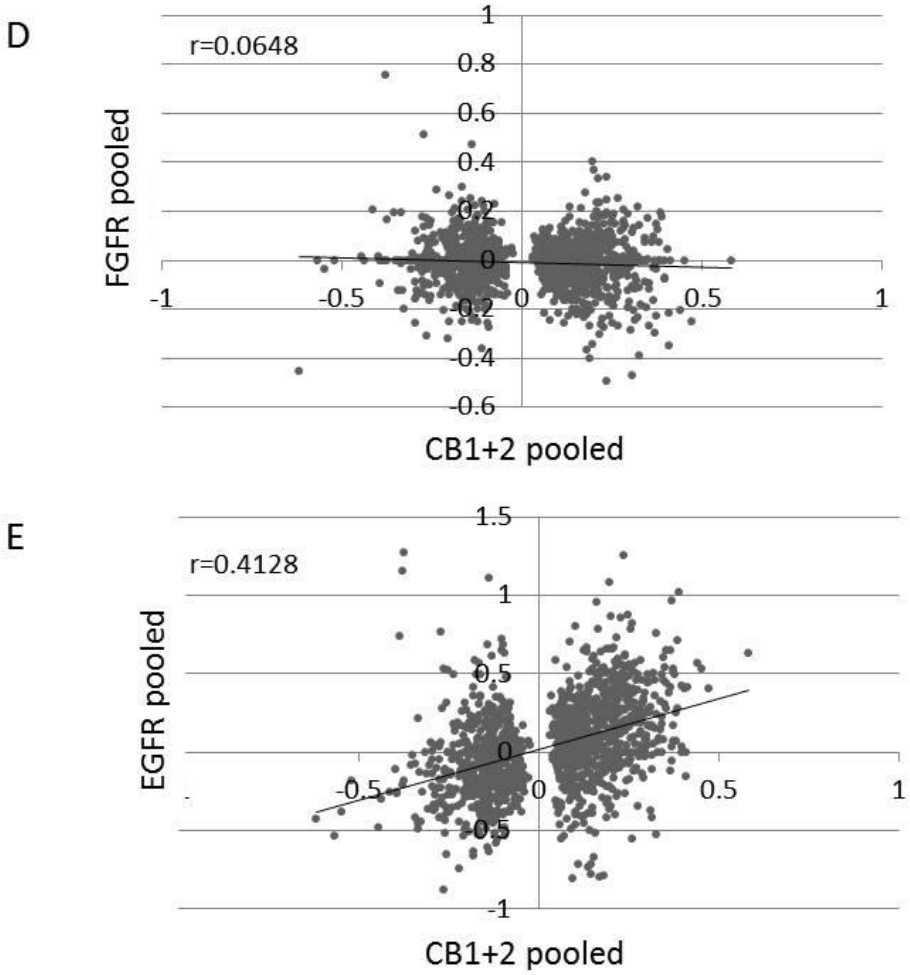


Figure 5.1: Correlations of transcriptional responses of Cor-1 cells to CB1 receptor, CB2 receptor, EGFR and FGFR antagonist treatments
Linear regression analysis of overlapping responses to (A) CB1 receptor antagonists AM251 and LY320 (both 1µM), (B) CB2 receptor antagonist AM630 and JTE-907 (both 1µM), (C) pooled CB1 receptor antagonists and pooled CB2 receptor antagonists, (D) Pooled CB1 and CB2 receptor antagonists and pooled EGFR antagonists AG1478 and PD168393 (both 100 nM), pooled CB1 and CB2 receptor antagonists and the FGFR antagonist PD173074 (500nM). A high degree of correlation is observed between both CB1 and CB2 receptor antagonists as well as between the pooled CB1 and CB2 receptor antagonists (A-C). A mild correlation (Pearson's correlation) was observed between the pooled CB1/2 receptor antagonists and the combined EGFR antagonists (E), whereas no correlation was observed between the pooled CB1/2 receptor antagonists and the FGFR antagonist (D).

	eCB	EGFR	FGFR
Number of experiments (n)	12	6	3
Down regulated responders (Average)	594 (-0.15)	369 (-0.54)	574 (-0.17)
Up regulated responders (Average)	768 (0.17)	2294 (0.67)	504 (0.19)
Highest responder downregulation	-0.61	- 1.53	-0.5
Highest responder up regulation	0.58	1.82	0.79
Total significant responders	1362	2663	1078

Table 5.1: Transcriptional response of Cor-1 cells to CB1 receptor, CB2 receptor, EGFR and FGFR antagonist treatments

Transcriptional responses of Cor-1 cells to different drug treatments were established by microarray analysis. The cells were treated with AM251, LY320, AM630, JTE-907 (all 1µM) and are displayed in this table as the combined eCB antagonists. EGFR antagonists AG1478 and PD168393 (both 100 nM) and the FGFR antagonist PD173074 (500 nM) were also combined and are displayed in the table. Treatment response was defined by 2(t-c)/(t+c), t being the average treatment and c being the average control signal (DMSO).

Again, a very strong correlation was observed when comparing the two EGFR antagonists (data not shown) and therefore responders were pooled for comparison with the CB1/2 receptor responders (Figure 5.1 E). A mild correlation was observed (r=0.4128), whereas the correlation coefficient between the FGFR inhibitor and eCB inhibitors does not show any correlation (Figure 5.1 D). These data do not support the hypothesis that FGFR signaling drives the eCB response as if this were the case the transcripts regulated by the eCB receptors would be expected to be regulated by the FGFR. The alternative hypothesis that the EGFR drives the eCB response is not disproven, and will be investigated further below.

Comparing the range of transcriptional response between eCB, EGFR and FGFR it is noticeable (Table 5.1), that the range and amount of responders is considerably different. While 1362 responders were significantly changed in the pooled CB1/2 receptor microarray sets twice as many responders were significantly changed when treated with EGFR antagonists. The range of responses is also different ranging between -0.61 and 0.58 for the eCB responder, - 1.52, and 1.82 for the EGFR responders and -0.5 and 0.79 for the FGFR responders. This shows that EGFR antagonist treatment results in a broader and more intense change in transcriptional response when compared with eCB antagonist treatment or FGFR antagonist treatment.

5.2.2. The effect of EFG, FGF and insulin on Cor-1 proliferation

Next we wanted to establish the relevance of EGF and FGF-2 for Cor-1 proliferation. Cor-1 cells were grown on gelatine coated 96-well plates in growth media, while the supplemented growth factors were adjusted depending on the experiment and live cell imaging using IncuCyte technology was used to visualise proliferation. Cor-1 cells were grown without FGF-2 supplementation, and a reduction in Cor-1 confluence was observed (Figure 5.2 A), but cells remain healthy and proliferating. The reduction of EGF below the usual 10ng/ml leads to a drastic reduction in Cor-1 proliferation (Figure 5.2 B).

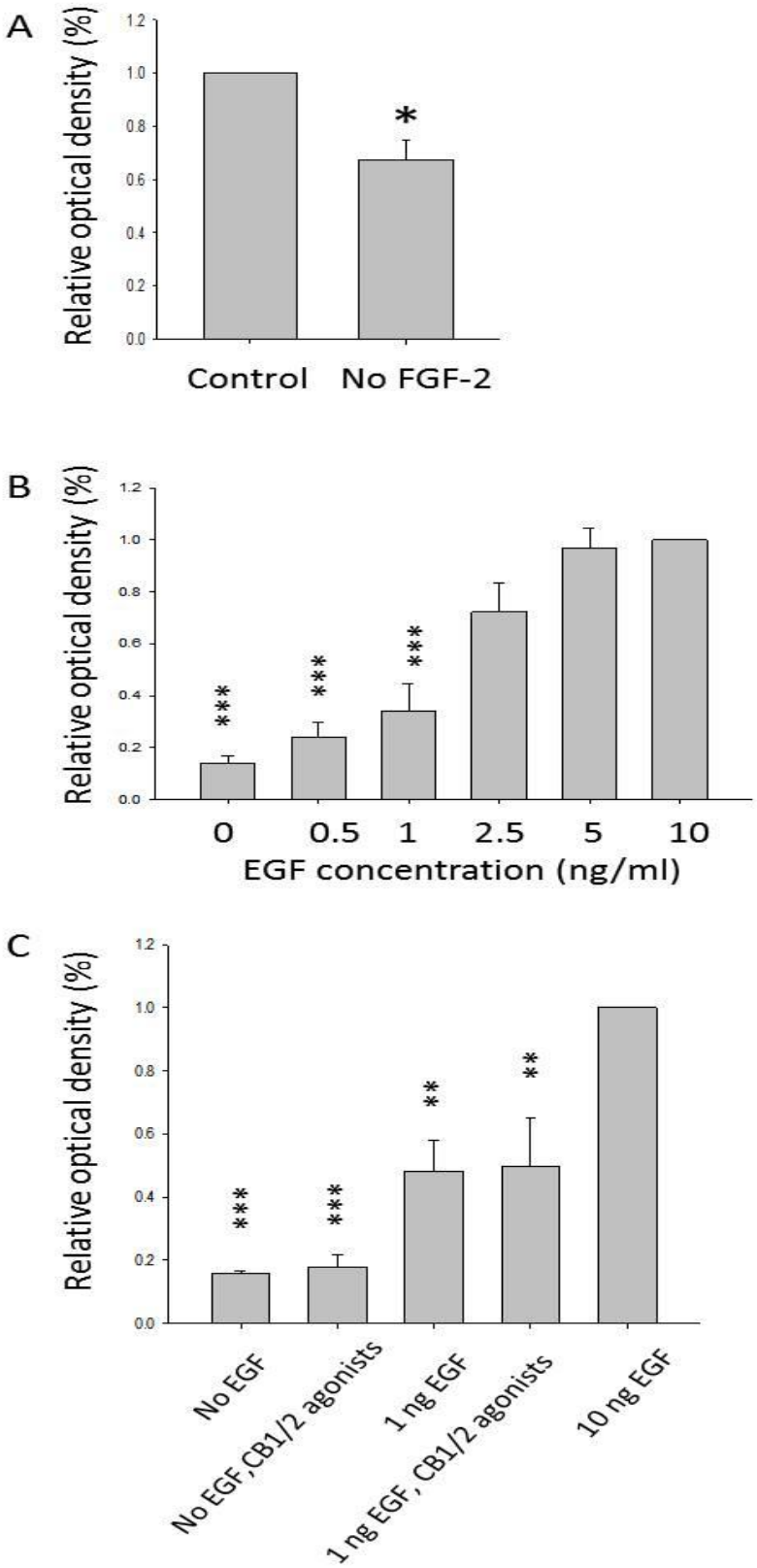

A
Relative optical density (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
No FGF-2
B
Relative optical density (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
0
0.5
1
2.5
5
10
EGF concentration (ng/ml)
C
Relative optical density (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
**
**
No EGF
No EGF,CB1/2 agonists
1 ng EGF
1 ng EGF , CB1/2 agonists
10 ng EGF

Figure 5.2: Cor-1 proliferation is reduced in the absence of EGF and FGF-2

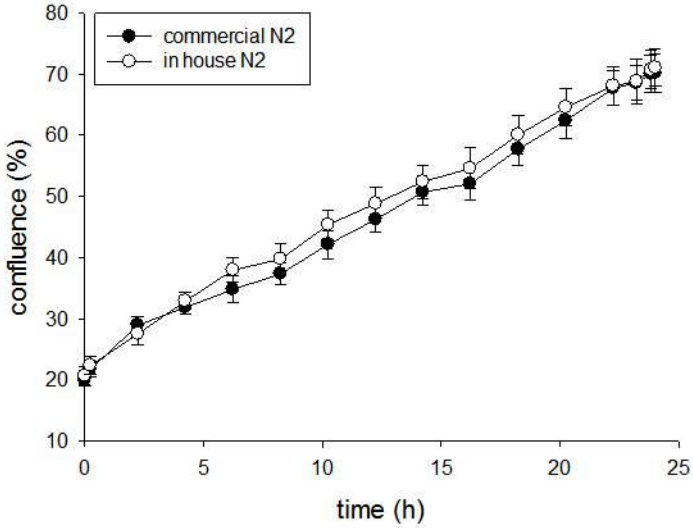
8,000 Cor-1 cells were grown in 96-well plates in growth media containing normal (10ng/ml) FGF-2 concentrations and normal (10ng/ml) EGF concentrations for the control, or in the absence of FGF-2 for 24h. A MTS cell proliferation assay was performed and the optical density relative to the control is displayed in this graph. A student t-test was performed and confirms a significance reduction in proliferation (A). 8,000 Cor-1 cells were grown in 96-well plates in growth media containing normal (10ng/ml) FGF-2 concentrations and normal (10 ng/ml) EGF concentrations for the control or in a range of EGF concentrations ± CB1+2 receptor agonists (1µM ACEA and 1µM JWH 133). A MTS cell proliferation assay was performed and the optical density relative to the control is displayed in this graph (B+C). The average of the means of three independent experiments ± SEM is presented (A-C). (B+C) Significance was calculated using anova analysis resulting in a p-value of 0.001 for the data presented in figure B and C. Significant differences according to post-hoc testing are indicated in the graph (Tukey test). Significance is indicated as the following:

$p< 0.05$ *, $P<0.01$ **, $p<0.001$ ***

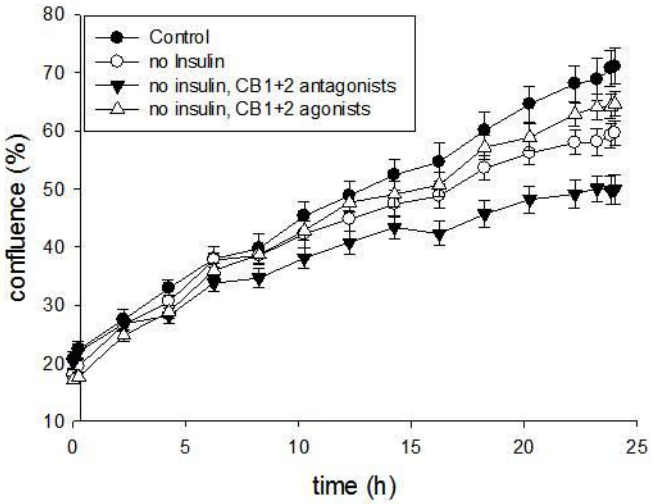
Based on the partial overlap in responders seen in Figure 5.1 between eCB and EGFR responders we next investigated if eCB agonists can boost Cor-1 proliferation in reduced EGF conditions as might be expected if eCB signaling was downstream from EGFR signaling. AM251 and JHW 133 (both at 1µM) were added to cells grown without or at 1ng/ml EGF, but no significant difference is detected between CB1+2 receptor agonist treated and untreated cells (Figure 5.2 C). This shows that eCB signaling cannot recover Cor-1 proliferation under low or no EGF conditions. While it is possible that EGF signaling drives eCB signaling in other contexts, this shows that it is unlikely to drive eCB signaling in the context of Cor-1 proliferation.

Next we investigated the effect of insulin withdrawal on Cor-1 proliferation (Figure 5.3). Insulin is part of the commercial N2 supplement added to the media to create the growth media. To vary insulin levels, an "in house" N2 was used and insulin was separately added. We compared Cor-1 proliferation using an IncuCyte live cell imaging. No difference was detected between the commercial and the in house N2 and cells looked healthy and proliferated at the same rate (Figure 5.3 A). Cor-1 cells were grown with in-house N2 containing insulin (Control) or no insulin and CB1/2 receptor agonists (0.5µM ACEA and JWH 133) or antagonists were added (1µM AM251 and JTE-907). Cor-1 cells were grown for 24h and live cell images are taken every 2h and the confluence was calculated using IncuCyte software. The area under the curve was calculated using an Excel macro and normalised to the control. The average of 3 experiments is shown in Figure 5.3 D demonstrating a significant reduction of confluence in cells grown without insulin. Confluence can be further reduced using CB1/2 receptor antagonists and can be restored to control confluence levels using CB1/2 receptor agonists. Therefore insulin might drive eCB signaling in the context of Cor-1 cell proliferation.

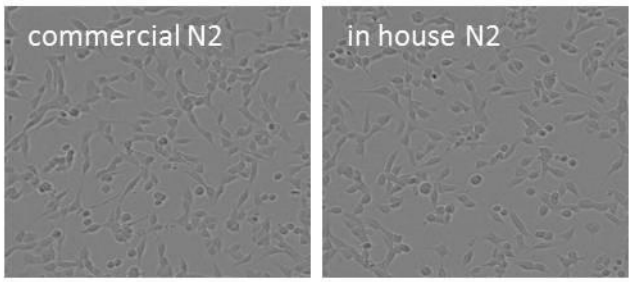
A

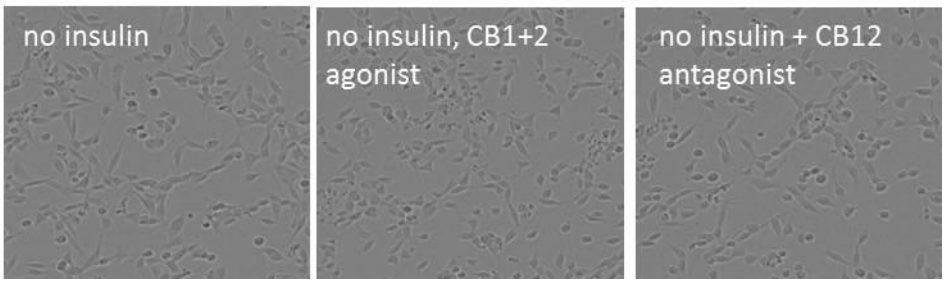

commercial N2
in house N2
confluence (%)
time (h)

B


Control
no Insulin
no insulin, CB1+2 antagonists
no insulin, CB1+2 agonists
confluence (%)
time (h)

C


commercial N2
in house N2


no insulin
no insulin, CB1+2 agonist
no insulin + CB12 antagonist

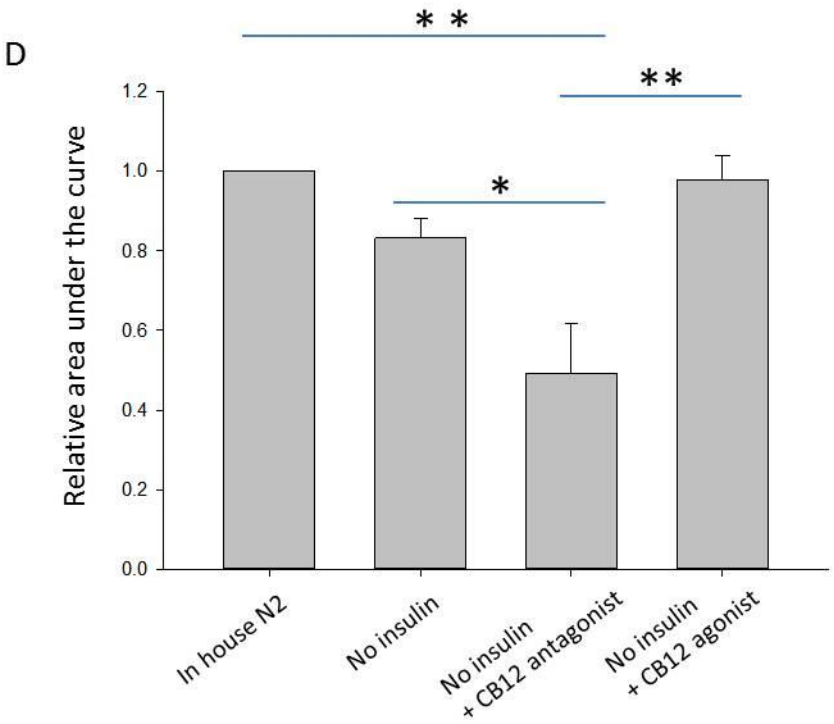


Figure 5.3: The effect of the absence of insulin on Cor-1 proliferation under CB1/2 receptor agonist or antagonist treatment

8,000 Cor-1 cells were seeded onto gelatine coated 96-well plates and in fully supplemented growth media and left to attach overnight. The media was carefully removed and growth media either containing the commercial or in house N2 media was added (A). In house media was identified to be equivalent to the commercial N2 supplement and is referred to as control in B. Cells were grown in the absence of insulin and additionally treated with CB1+2 receptor antagonists (0.5µM AM251 and JTE-907) or CB1+2 receptor agonists (1µM ACEA and JWH 133). Cell growth was filmed for 24h and monitored using IncuCyte live cell imaging. Images taken after 24h are presented in C. The area under the curve was calculated using an Excel macro software and is sown in D as the mean of three independent experiments ± SEM. The significance was calculated using the anova resulting in a p-value of 0.004 and Tukey post-hoc testing was used to determine the significance between groups.

P< 0.01 **

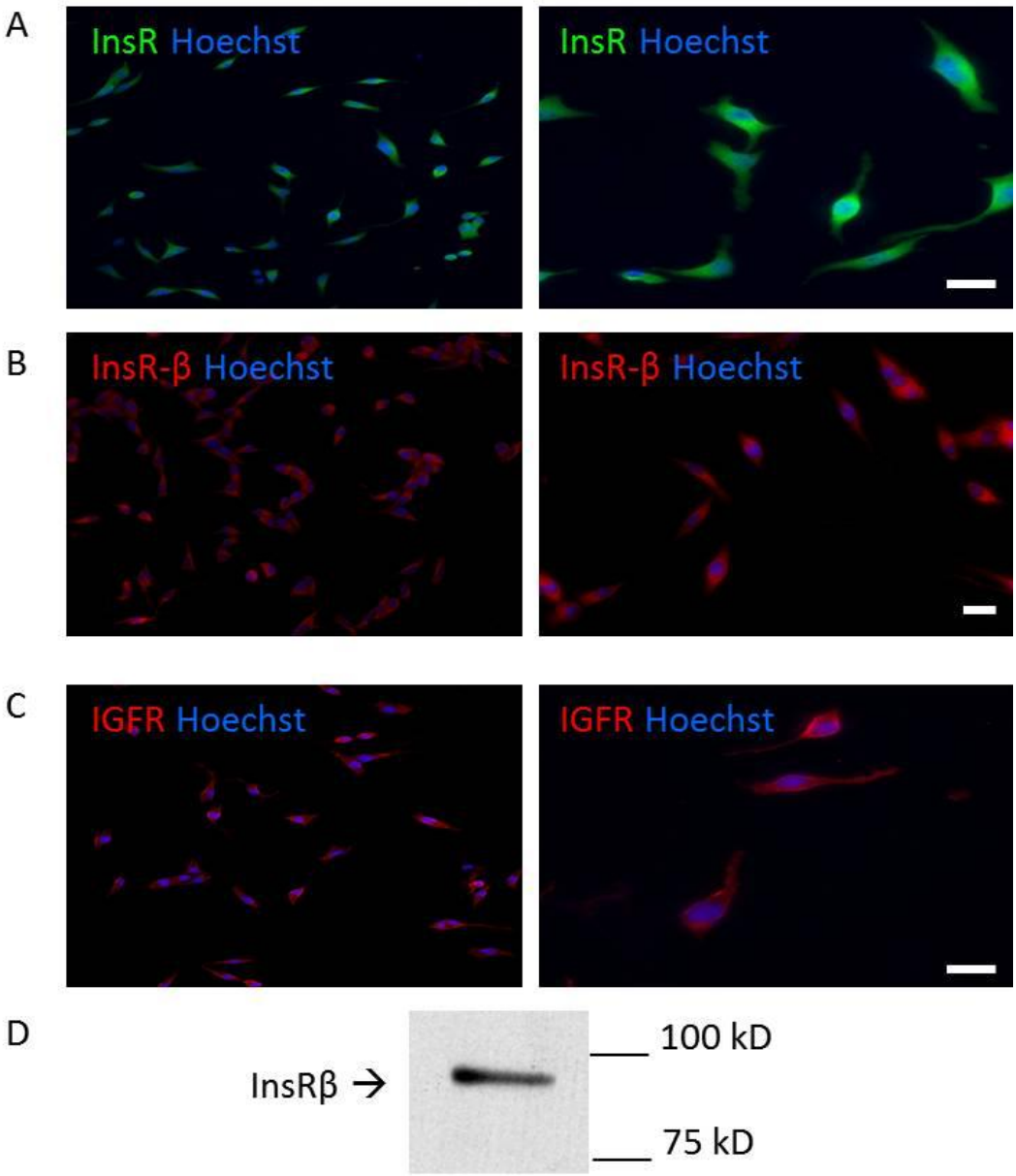


Figure 5.4: Expression of InsR and IGFR

Cor-1 cells were grown on glass cover slips coated with gelatine at 20,000 cells/ well in growth media in 4-well plates. The cells were left to attach overnight and fixed using PFA before being used for immunocytochemistry. An anti-InsR antibody (A), an anti-InsR β chain antibody (B) and an anti-IGFR antibody (C) were used as well as the nucleus dye Hoechst. Cor-1 cells were grown under normal growth conditions, their protein was extracted and used for western blot analysis. An anti-InsR β chain antibody (D) detects a band at the expected MW of 95 kDa.

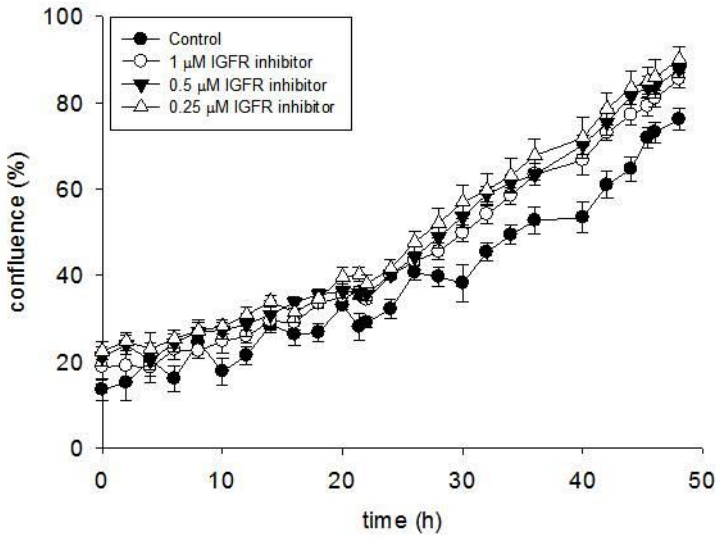
To further investigate insulin signaling, we wanted to establish if the insulin signaling related receptors are present in Cor-1 cells. We identified the insulin receptor (InsR), the β chain of InsR and the IGFR to be present by immunocytochemistry and/or western blot analysis (Figure 5.4).

H-1356 is an inhibitory IGF-1 peptide analog inhibiting the IGFR. It was used at concentrations between 0-1μM in an IncuCyte live cell imaging based Cor-1 proliferation assay (Figure 5.5). No fundamental changes in cell proliferation or shape were observed. It therefore appears that the IGFR is not involved in regulation of proliferation in Cor-1 cells.

AG1024 inhibits the IGFR as well as InsR activity. 10nM has been shown to inhibit cancer cell proliferation (Wen et al., 2001). It was used at concentrations between 0-0.5μM. At higher concentrations it induced cell death, but at lower concentrations it reduced cell proliferation significantly (Figure 5.6). This result supports the result seen in Figure 5.3, where cells showed reduced proliferation upon insulin withdrawal.

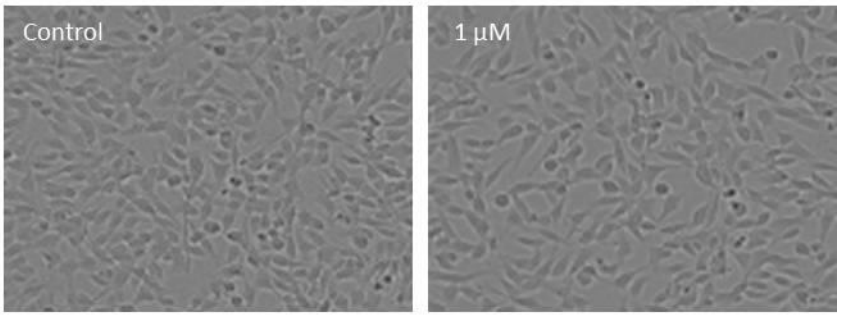
In summary, based on the lack of a transcriptional response overlap between CB1/2 receptor signaling and FGFR signaling (Figure 5.1) we conclude that FGFR is not upstream of the CB1/2 receptor in Cor-1 cells. Furthermore, Cor-1 proliferation only is reduced by ~30% in the absence of FGF-2, while Cor-1 cell cannot proliferate in the absence of EGF (Figure 5.2). The transcriptional response between the different CB1 and CB2 receptor drugs showed a remarkably strong correlation. A smaller overlap between the transcriptional response of CB1/2 receptor signaling and EGFR receptor was identified (Figure 5.1). This might indicate that they share a common downstream pathway. Based on the micro array data, the mammalian target of rapamycin (mTOR) pathway was identified as a promising downstream pathway (personal communication with Dr Gareth Williams) and was further investigated (see below).

A



IGFR inhibitor treated for 48 hours

B



C

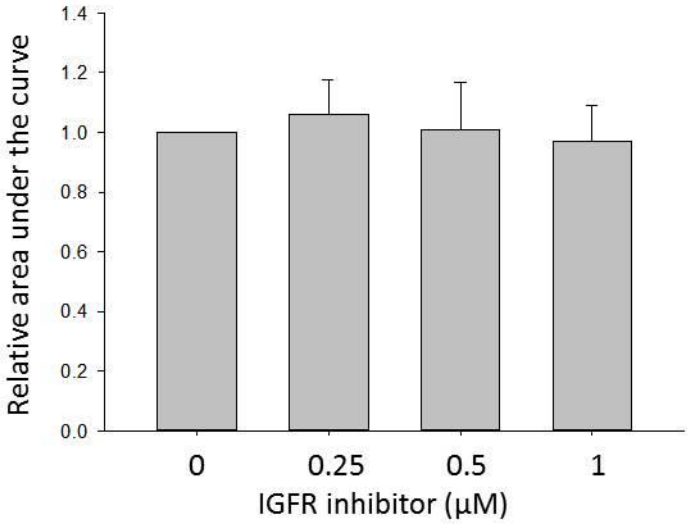


Figure 5.5: The effect of IGFR inhibitor on Cor-1 proliferation
8,000 Cor-1 cells were seeded onto gelatine coated 96-well plates and in fully supplemented growth media and left to attach overnight. H-1356 is an IGF-1 inhibitory analog acting as an antagonist to the IGFR and it was added at 0-1µM to the cells and cell proliferation was observed using IncuCyte live cell imaging and is displayed as confluence in A. Representative images taken at 24h of treatment are shown in B and the area under the curve was calculated using an Excel macro software and is shown relative to the control as the mean ± SEM from three independent experiments (C). Significance is calculated using the student t-test and no significant difference was found.

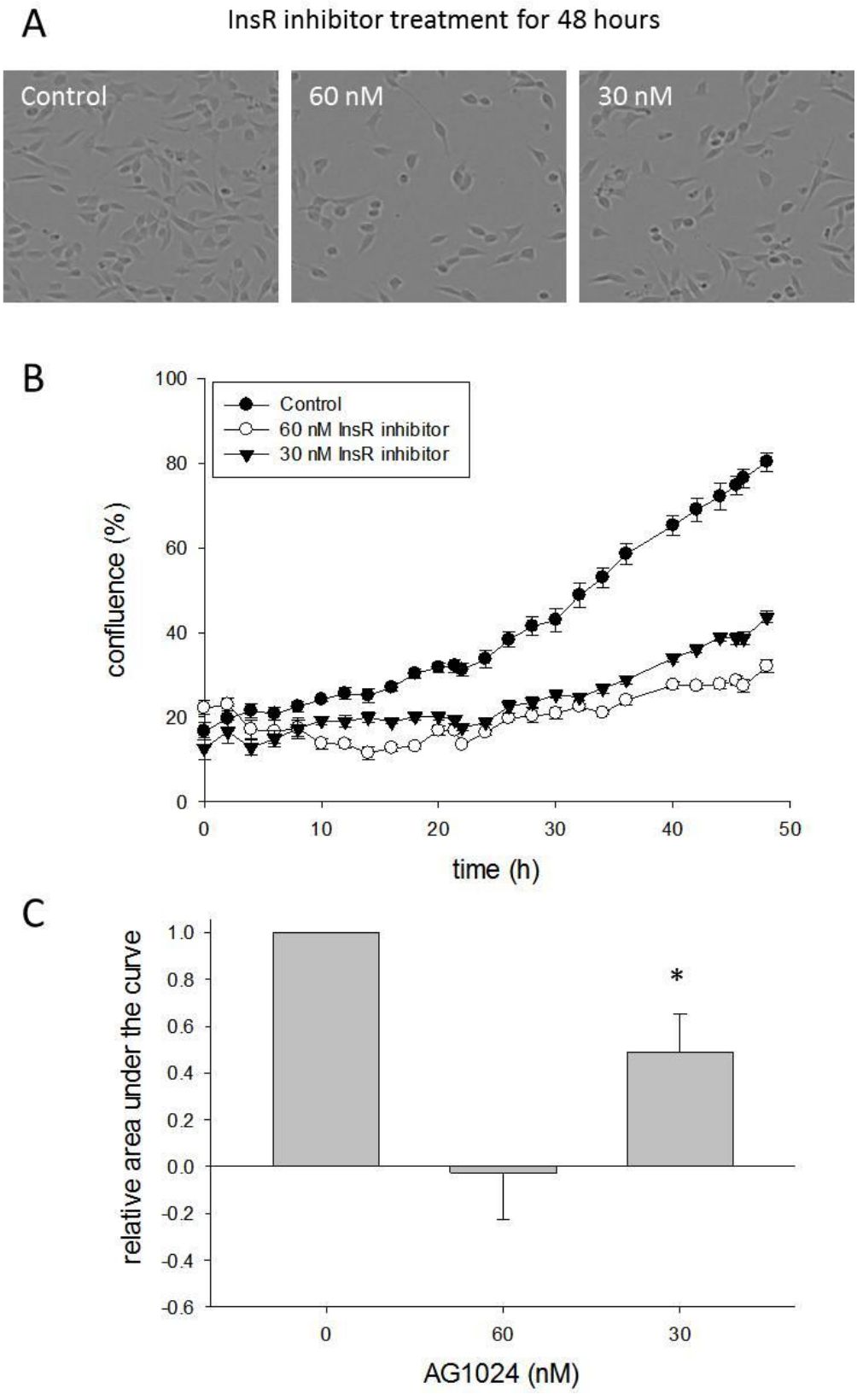

A
InsR inhibitor treatment for 48 hours
Control
60 nM
30 nM
B
Control
60 nM InsR inhibitor
30 nM InsR inhibitor
confluence (%)
time (h)
C
relative area under the curve
AG1024 (nM)
*

Figure 5.6: The effect of InsR/IGFR inhibitor on Cor-1 proliferation
8,000 Cor-1 cells were seeded onto gelatine coated 96-well plates and in fully supplemented growth media and left to attach overnight. 0-60nM InsR/IGFR inhibitor was added to the cells and cell proliferation was observed using IncuCyte live cell imaging and is displayed as confluence in A. Representative images taken at 24h of treatment are shown in B and the area under the curve was calculated using an Excel macro software and is shown relative to the control as the mean ± SEM of three independent experiments (C).

P< 0.05 *

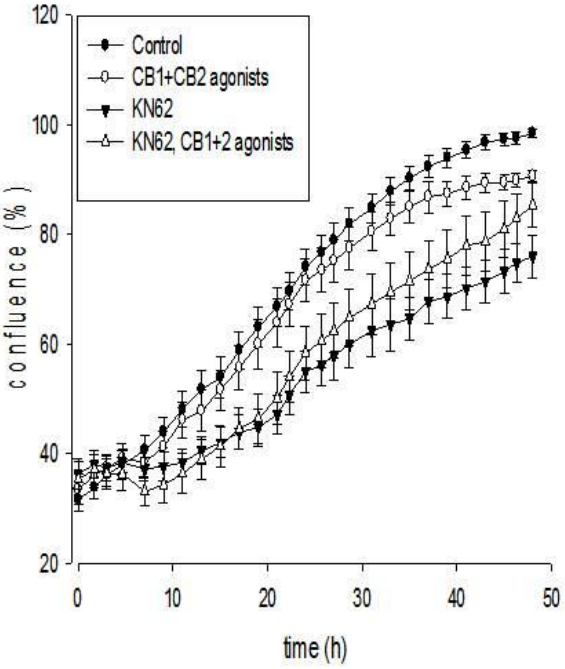
Based on the observation that eCB signaling can partially compensate for loss of insulin signaling, I would suggest that this looks like the best candidate to be the growth factor that generates eCB tone, however due to time constraints I have been unable to obtain direct evidence to support this hypothesis, and other additional pathways need to be upstream driving the CB1/2 receptors as well since Cor-1 proliferation can be further reduced by eCB inhibitors in the absence of insulin (Figure 5.3). In this context of Cor-1 proliferation the InsR is a more likely candidate than the IGFR to be involved in this process.

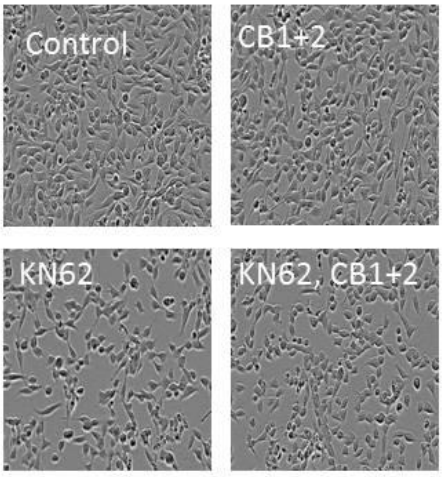
5.2.3. Investigation of kinases suspected to be upstream of the CB1/2 receptors and influencing CB1/2 receptor signaling

Growth factor receptor signaling cascades often include a series of phosphorylation events via kinases as has been well established for many pathways such as the mitogen-activated protein kinase (MAPK) signaling pathway (reviewed in McCubrey et al., 2007). Combining bioinformatic approaches, existing experimental data and our own unpublished observations we predict that DAGLα is phosphorylated (for more information see below). Especially PKA and PKC have been identified as strong candidates to phosphorylated DAGLα. Based on this I wanted to test a number of kinases to see if the activity was required upstream of the CB1/CB2 receptor response. A straightforward logic was applied – if a kinase inhibitor could block proliferation and this could be recovered by CB1/CB2 receptor agonists, then that kinase might be considered as a candidate for generating eCB tone, perhaps via phosphorylation and activation of DAGL.

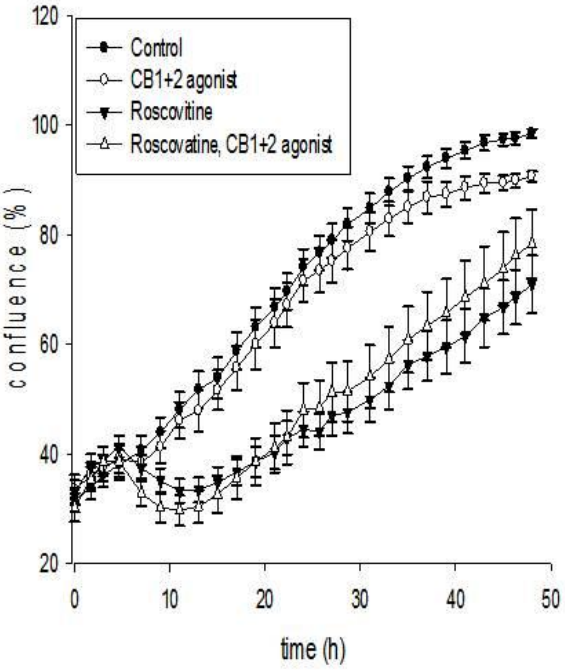
Bioinformatic tools were used to identify phosphorylation sites on the hDAGLs to predict kinases that might be responsible for this. PhosphoSitePlus (www.phosphosite.org) for example is an online resource giving comprehensive collections of data on post translational modifications including phosphorylation while NetPhospho 2.0 is another bioinformatics tool to identify phosphorylation sites and hDAGLα was investigated. A high score of 0.998 (1 being the highest score possible) for S402 was revealed for hDAGLα indicating a very high likelihood for phosphorylation of this amino acid.

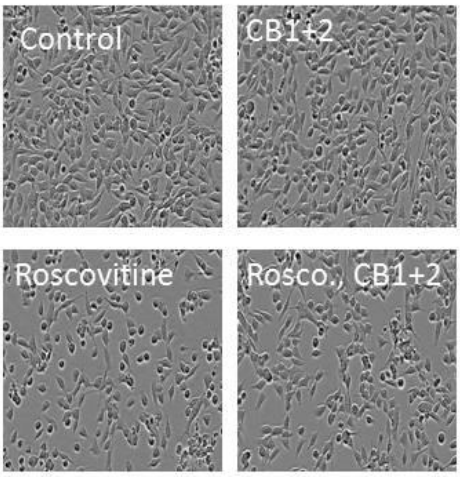
A


Control
CB1+CB2 agonists
KN62
KN62, CB1+2 agonists
confluence (%)
time (h)

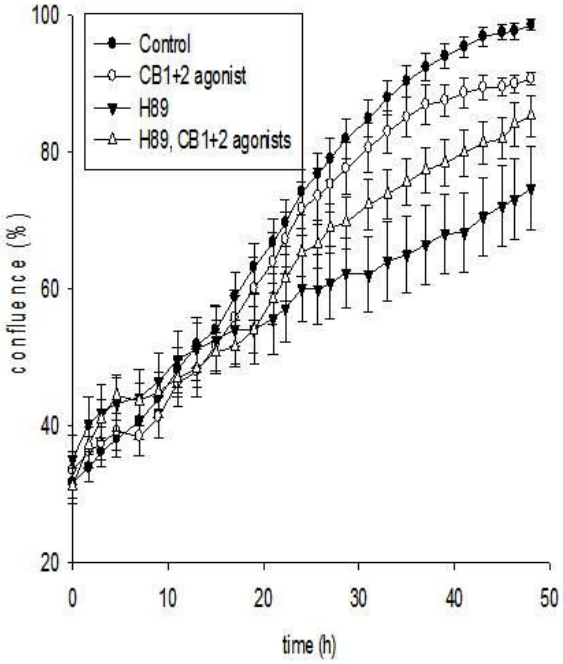

Control
CB1+2
KN62
KN62, CB1+2

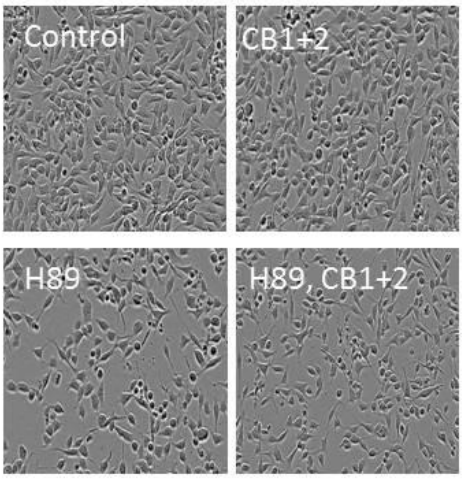
B


Control
CB1+2 agonist
Roscovitine
Roscovatine, CB1+2 agonist
confluence (%)
time (h)

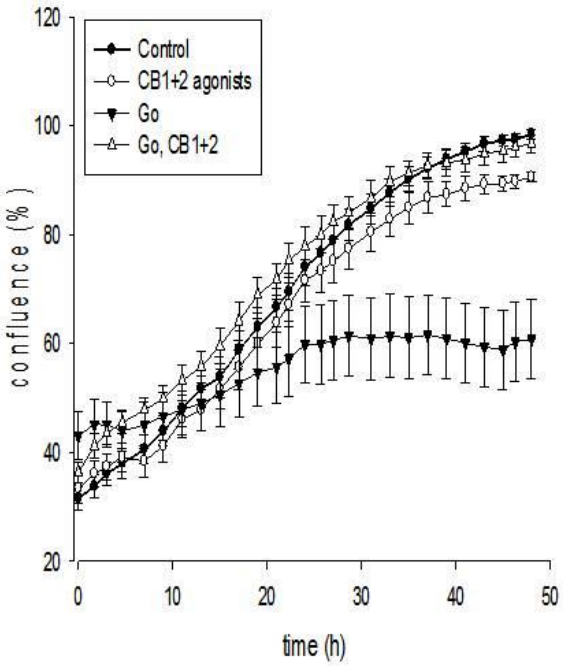

Control
CB1+2
Roscovitine
Rosco., CB1+2

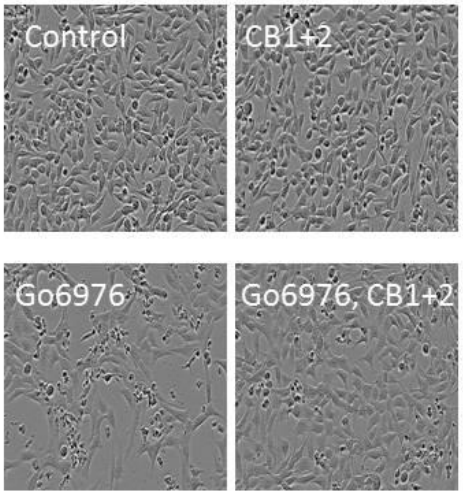
C


Control
CB1+2 agonist
H89
H89, CB1+2 agonists
confluence (%)
time (h)


Control
CB1+2
H89
H89, CB1+2

D


Control
CB1+2 agonists
Go
Go, CB1+2
confluence (%)
time (h)


Control
CB1+2
Go6976
Go6976, CB1+2

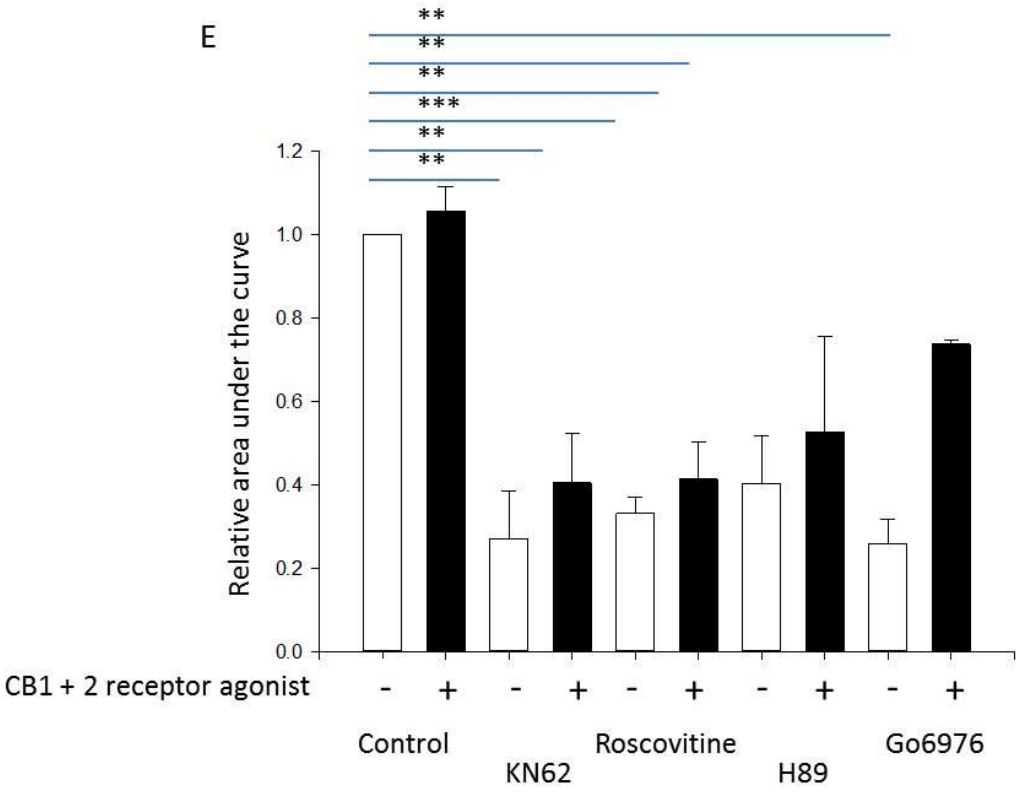


Figure 5.7: The effect of kinase inhibitors on Cor-1 proliferation with or without CB1+2 receptor agonist treatment

8,000 Cor-1 cells were seeded onto gelatine coated 96-well plates and left to attach overnight in growth media. The next day the cells were treated with 1µM KN62 (A), 1µM Roscovitine(B), 5µM H89 (C) or 1µM Go 6976 (D); all kinase inhibitors ± 1µM ACEA+JWH-133. Cor-1 cell proliferation was observed for 48h using IncuCyte live cell imaging and the confluence covered by the cells is displayed as well as pictures at 48h of treatment (A-D). The area under the curve was calculated using a macro excel software and the data is presented normalised to the control as the average of the mean of three independent experiments ± SEM (E). Significance compared to the control was calculated (anova, p-value 0.001) and significance according to Tukey testing is indicated in the figure.

P < 0.05*, P< 0.01 **, P< 0.001 ***

DAGLα and DAGLβ were investigated using these and other tools to identified phosphorylation sites (Figure 7.2). Furthermore, we predict that DAGLα phosphorylation is important for its active configuration status based on comparisons with other α/β hydrolases (Reisenberg et al., 2012).

Amongst other databases the Human Protein reference database allows recognition of kinase substrate sites and helped to identify possible kinases. Furthermore, PKA was shown to positively regulate phosphorylation of DAGL purified from brain microsomes (Perez Roque et al., 1998; Rosenberger et al., 2007). PKC and PKA have been shown to increase 2-AG synthesis, most likely via a DAGL-dependent mechanism (Vellani et al., 2008). PKA, PKC, CamK II, and CDK have been identified as good candidates to investigate regarding DAGL phosphorylation based on the data mentioned above and observations obtained with a CB1 receptor activation assay (Emma Williams, unpublished observations).

Kinase inhibitors for CamK II (KN62), CDK 1, 2, 5 (Roscovitine), PKA (H89) and PKC (Go6976) were investigated in the context of DAGLα activity. Their effect on Cor-1 proliferation was investigated using live cell imaging with the IncuCyte. We wanted to establish if the kinase inhibitors have an effect on Cor-1 proliferation and if this effect can be rescued using CB1/2 receptor agonists. This would be a good indication that a phosphorylation event upstream of the CB1/2 receptors influenced receptor signaling potentially via 2-AG synthesis. Cor-1 cells were treated with 0-5μM KN62, 0-10μM roscovitine, 0-5μM H89 and 0-1μM Go 6976 and cell confluence was measured for 48h using IncuCyte live cell imaging and software. All investigated kinase inhibitors inhibited Cor-1 proliferation in a dose dependent manner (data not shown). Next we wanted to investigate if CB1/2 receptor agonism could reverse this inhibition. Cor-1 cells were treated with 1μM KN62, 1μM roscovitine, 5μM H89 or 1μM Go6976 ± CB1/2 receptor agonists (1μM ACEA and 1μM JWH-133) and grown for 48h under these conditions. Proliferation was observed using IncuCyte live cell imaging and representative confluence curves are shown in Figure 5.7 (A-D).

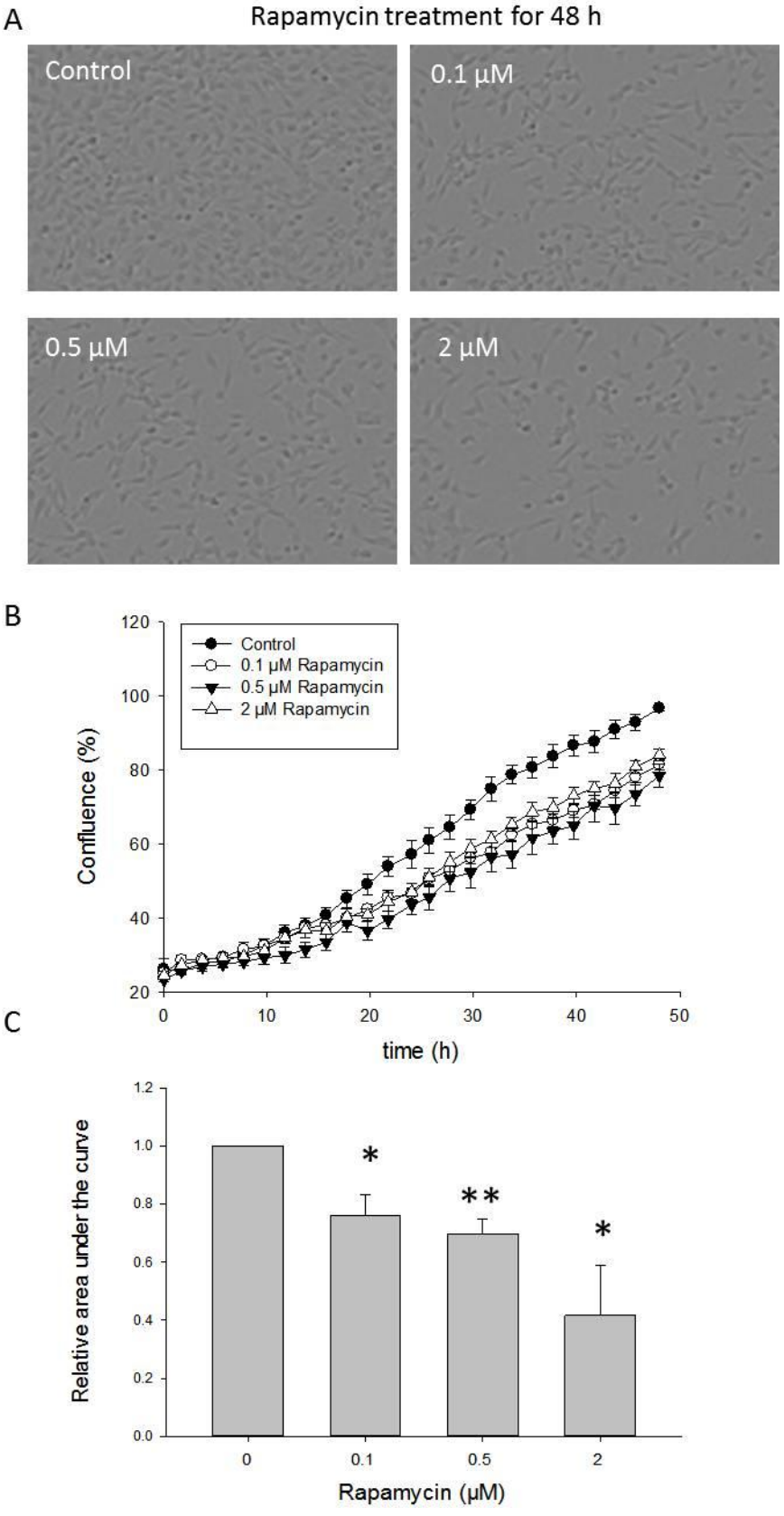

A
Rapamycin treatment for 48 h
Control
0.1 µM
0.5 µM
2 µM
B
Control
0.1 µM Rapamycin
0.5 µM Rapamycin
2 µM Rapamycin
Confluence (%)
time (h)
C
Relative area under the curve
Rapamycin (µM)
*
**
*

Figure 5.8: The mTOR inhibitor rapamycin inhibits proliferation of Cor-1 cells

Cor-1 cells were seeded at a density of 8,000 cells/well and left overnight to attach. The cells were then treated with 2µM , 0.5µM or 0.1µM rapamycin and two phase-contrast pictures were taken every 2h for 48h using IncuCyte imaging. Pictures taken after 48h of treatment are shown in (A). Confluence of the cells was calculated by the built-in IncuCyte software and a representative proliferation curve can be seen in B. The area under the curve was calculated using an Excel Macro and results from 3 independent experiments were pooled. The mean ± SEM is displayed. Significance was calculated using the anova and significance change in comparison with the control is indicated as stated below (C).

* $p<0.05$, ** $p<0.01$, *** $p< 0.001$

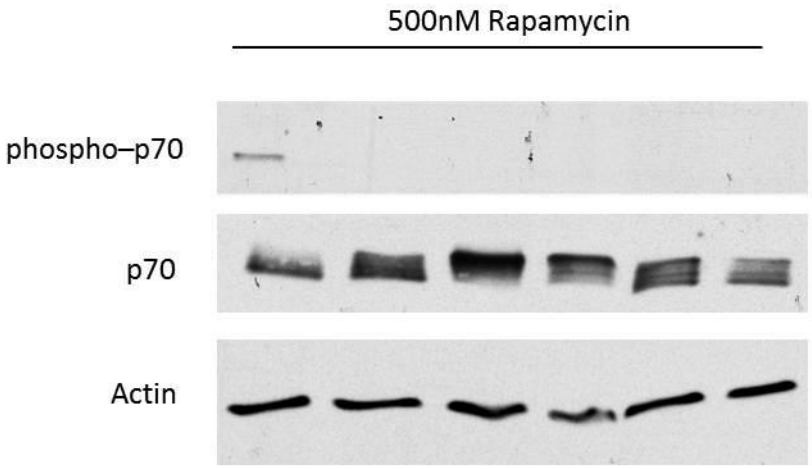
In the cases of KN62 (Figure 5.7 A) and roscovitine (Figure 5.7 B) the additional treatment with CB1/2 receptor agonists did not increase proliferation. H89 and Go6976 treatment reduced Cor-1 confluence and this could be increased by using CB1/2 receptor agonists. In the case of H89, the confluence remains lower than under control conditions, while in the case of Go6976, the confluence was restored to control conditions if treated with Go6976 and CB1+2 receptor agonists (Figure 5.1 C+D). It has to be mentioned that the cell morphology is changed by Go6976 treatment.

The area under the curve was calculated using an Excel Macro, normalised to the control and data from 3 independent experiments is presented in Figure 5.7 E. Treatment with all kinase inhibitors significantly decreased the relative area under the curve as calculated by using the student t-test and no change in proliferation was observed using KN62 or roscovitine. The treatment with H89 plus CB1+2 receptor agonists led to an enhanced Cor-1 proliferation which is a not significant trend. Only the inhibition of proliferation and/or change in morphology induced by Go6976 could be significantly increased by CB1/2 receptor agonist treatment. This might indicate a role of PKC in eCB signaling upstream of the CB1 and/or CB2 receptors. To test if this effect is a DAGL related effect further experiments are needed. One possibility would be via the use of phospho specific DAGLα antibodies which are not commercially available so far. Therefore our lab created 5 possible DAGLα-phospho antibodies and these as well as commercially available, generic kinase substrate antibodies were tested (data not shown). While the generic kinase substrate antibodies were not suitable, further experiments are needed to establish the suitability of the phospho-DAGLα antibodies. Furthermore, an assay to investigate DAGL activity has been published recently (Pedicord et al., 2011) and was tested for its suitability in combination with Cor-1 cells in Chapter 6.

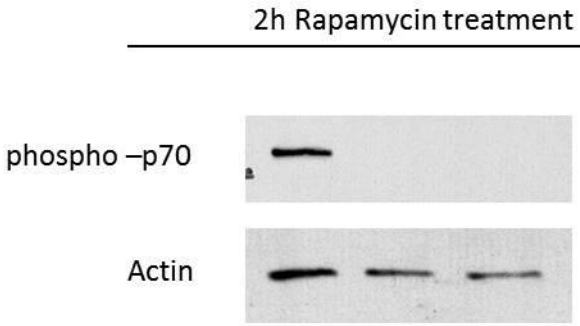
5.3. Is mTOR/p70 S6 kinase downstream of EGFR, FGFR and/or eCB signaling?

As mentioned earlier an overlap of transcriptional responds between CB1/2 receptor and EGFR inhibition was observed.

A **Time course of mTOR antagonist (Rapamycin) treatment**



B **(Rapamycin) treatment at different drug concentrations**



C

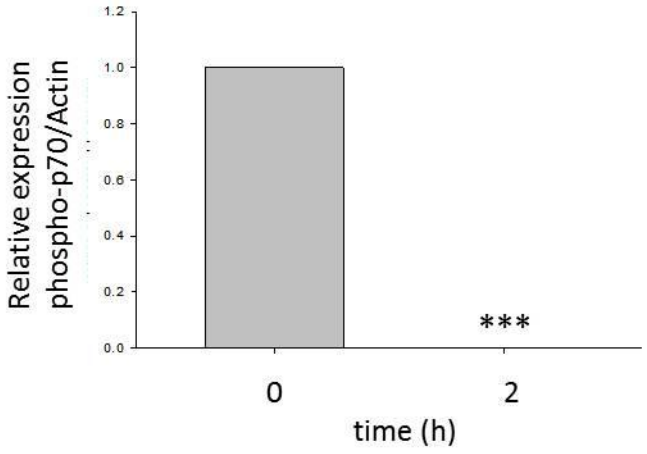


Figure 5.9: Rapamycin blocks p70 S6 kinase phosphorylation in Cor-1 cells

Cor-1 cells were grown in growth media until 70-75% confluence was reached. Cells then were then treated with 500 nM rapamycin for 0-4 h (A) or 2h with 0–500 nM rapamycin (B). The proteins were extracted and used for western blot analysis. p70-S6 Kinase 1 (p70) is a well established downstream target of mTOR and PI3K pathway. The phosphorylated state of threonine 389 is critical for kinase activity (phospho-p70) (A). The experiment was repeated 3 times for the 2h point with 500 nM rapamycin and the band intensity for phospho-p70 was analysed using ImageJ. The data was normalised to the control and is presented in C. The mean ± SEM of three independent experiments is displayed and significance was established using the student t-test.

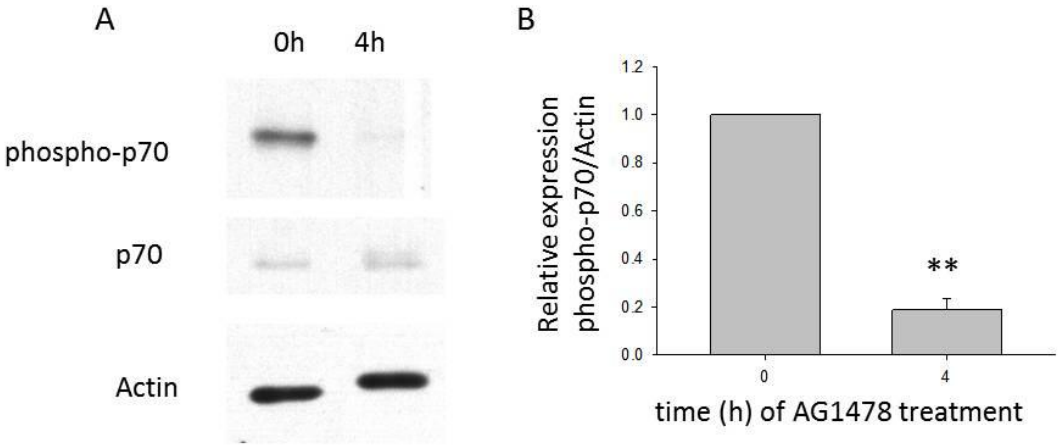
*** P< 0.001

While the correlation was not strong enough to account for the EGFR signaling driving eCB signaling in Cor-1 cells, it opened up the possibility that they might share a common downstream signaling hub. The mTOR pathway was identified as a promising candidate based on the acquired microarray data (personal communication Dr Gareth Williams,). This is also supported by recent publications linking the mTOR pathway to eCB signaling (Gomez et al., 2011; Keimpema et al., 2011; Palazuelos et al., 2011).

mTOR signaling is linked to a wide range of extra- and intracellular signaling mechanisms (Laplante and Sabatini, 2009). Growth factor signaling via the EGFR or IGFR as well as insulin, nutrients or stress is known to regulate mTOR signaling and the most common signaling pathway is through the PI3K/Akt pathway. The EGFR is known to signal via AKT/mTOR especially in carcinoma cells and other cancer cells (Han et al., 2006; O'Reilly et al., 2006; Freudlsperger et al., 2010). Furthermore, the mTOR/p70 S6 kinase (in Figures referred to as p70) pathway has been demonstrated to be modulated by CB1 receptor signaling in the mouse hippocampus (Puighermanal et al., 2009). Moreover, CB1/2 receptor agonism has been shown to increase oligodendrocyte differentiation via the mTOR and Akt signaling pathway (Gomez et al., 2011). Considering the partial overlap of transcriptional responses seen in the eCB and EGFR microarrays (Figure 5.1) and the previously mentioned findings, we wanted to investigate if EGFR and the CB receptors signal via the mTOR pathway.

We first wanted to confirm the role of mTOR signaling in Cor-1 cell proliferation, by using the mTOR inhibitor rapamycin (Figure 5.8). Rapamycin was used at concentrations between 0 and 2µM in an IncuCyte base Cor-1 cell proliferation assay and was seen to inhibit cell proliferation. Moreover, the relative area under the curve was calculated for three independent experiments and rapamycin significantly inhibited the relative area under the curve even at the lowest used rapamycin concentration of 0.1µM (Figure 5.8 B) while Cor-1 cells looked healthy even at the highest drug concentration (Figure 5.8 A).

EGFR inhibitor treatment (AG1478)



FGFR inhibitor treatment (PD173074)

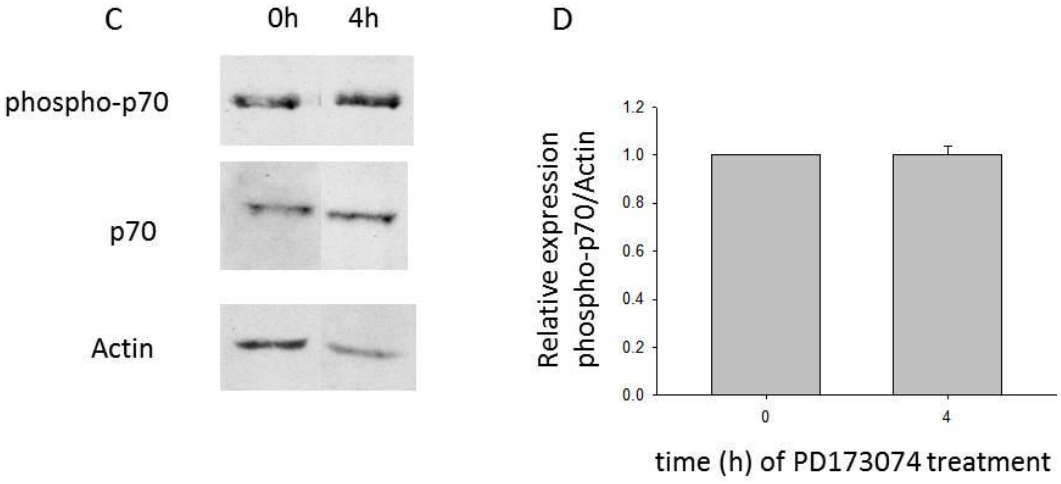


Figure 5.10: Influence of EGFR inhibitor (AG1478) and FGFR inhibitor (PD173074) on p70 S6 kinase phosphorylation in Cor-1 cells

Cor-1 cells were grown in growth media until 70-75% confluence was reached. Cells then were then treated with 100nM EGFR inhibitor AG1478 (A,B) or 500nM FGFR inhibitor PD173074 (C,D) for 0 or 4 h. The proteins were extracted and used for western blot analysis. p70-S6 kinase (p70) is a well established downstream target of mTOR and PI3K pathway. The experiments were repeated 3 times and the band intensity for phospho-p70 was analysed using ImageJ. The data was normalised to the control and is presented in in B and D as the mean ± SEM. Significance was established using the student t-test and is indicated as stated below.

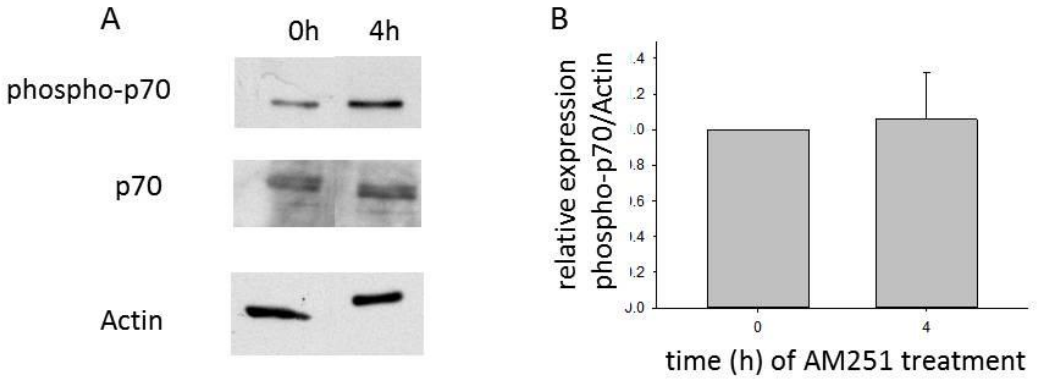
** P< 0.01 student t-test

The p70 ribosomal S6 kinase is a downstream target of mTOR and is activated by mTOR through phosphorylation and the mTOR/p70 S6 kinase pathway furthermore has been linked to cell proliferation (Wing et al., 2005; Varma and Khandelwal, 2007). To test if mTOR phosphorylates p70 S6 kinase in Cor-1 cells, we grew Cor-1 cells in the presence of 500nM rapamycin for 0-4h (Figure 5.9 A) or at different rapamycin concentrations between 0 and 0.5µM (Figure 5.9 B). Western blot analysis was performed and an anti-p70 S6 kinase (p70) and anti-phospho-p70 S6 kinase (phospho-p70) antibody was used to observe the influence of rapamycin treatment on the protein and its phosphorylation. While p70 S6 kinase levels remain even, its phosphorylation detected by phospho-p70 is not observed between 0.5-4h of rapamycin treatment. We therefore demonstrated that p70 S6 kinase is a downstream target of mTOR in Cor-1 cells and furthermore p70 S6 kinase phosphorylation, which is required for its activation, can be inhibited with the mTOR inhibitor rapamycin.

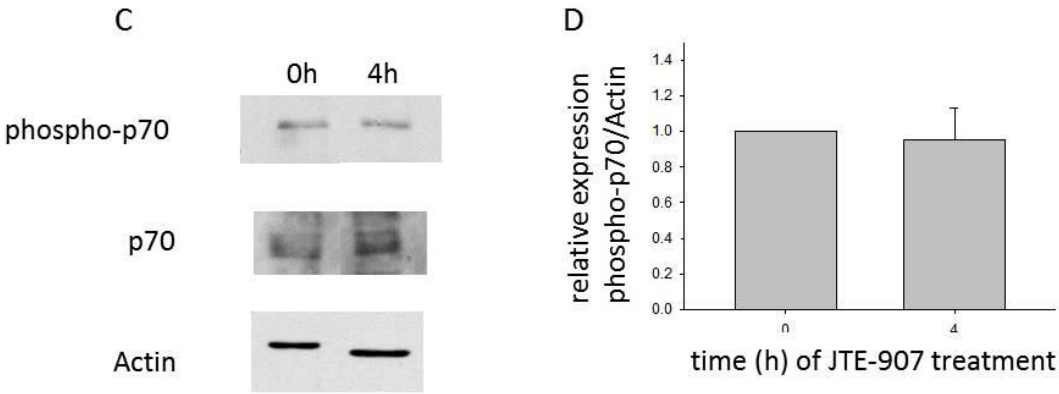
Next, we wanted to establish the effect of EGFR or FGFR treatment on the state of p70 S6 kinase. To test this, we treated Cor-1 cells with the EGFR inhibitor AG1478 or FGFR inhibitor PD173074 (Figure 5.10). We extracted the proteins and used them for western blot analysis. The drug treatment and protein extraction were carried out and samples were provided by Dr Philipp Sütterlin. Anti-p70 and anti-phospho-p70 antibodies were used to detect p70 S6 kinase and its phosphorylated form. While p70 S6 kinase remained even with both treatments (Figure 5.10 A, C), phospho-p70 levels were significantly reduced after 4 h EGFR inhibitor treatment (Figure 5.10 A, B), but unchanged by FGFR inhibitor treatment (Figure 5.10 C, D). This indicates that mTOR/p70 S6 kinase is downstream of the EGFR.

After establishing the relationship between EGFR and p70 S6 kinase activation in Cor-1 cells we next wanted to investigate if there is a relationship between eCB signaling and p70 S6 kinase activation. To do so, we treated Cor-1 cells with AM251, JTE-907 or both (all at 1µM) for 4h before extracting the proteins and using them for western blot analysis (Figure 5.11). No significant change in phospho-p70 levels was observed when treating the cells with AM251 and/or JTE-907. p70 S6 kinase levels remained even under all conditions.

CB1 receptor antagonist (AM251)



CB2 receptor antagonist (JTE-907)



CB1/2 receptor antagonist (AM251 plus JTE-907)

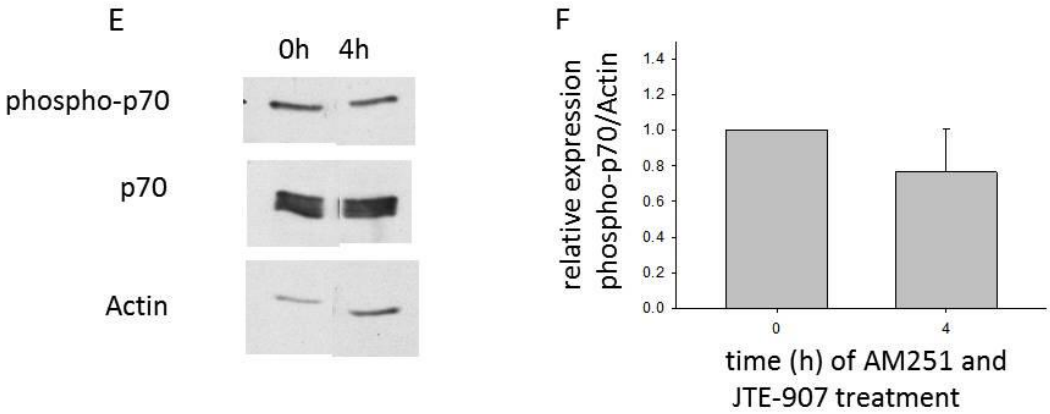


Figure 5.11: Influence of CB1 receptor antagonist (AM251) and CB2 receptor antagonist (JTE-907) on p70 S6 kinase phosphorylation in Cor-1 cells

Cor-1 cells were grown in growth media until about 70-75% confluence was reached. Cells then were then treated with 1µM AM251 (A, B) or 1µM JTE-907 (C,D) or both (E,F) for 0 or 4 h. The proteins were extracted and used for western blot analysis. p70-S6 Kinase 1 (p70) is a well established downstream target of mTOR and PI_3K pathway. The experiments were repeated 3 times and the band intensity for phospho-p70 was analysed using ImageJ. The data was normalised to the control and is presented in in B ,D E as the mean ± SEM of three independent experiments. Significance was calculated using the student t-test and no significance was detected (B, D, F).

This shows that even though the mTOR/p70 S6 kinase pathway is downstream of EGFR signaling in Cor-1 cells, it is not downstream of eCB signaling.

5.4. Conclusions and discussion

The eCB system has a role in neurogenesis and NSC proliferation (Goncalves et al., 2008; Oudin et al., 2011a). While the FGFR has been shown to be upstream of eCB signaling in another context (Williams et al., 2003) it is not known what drives eCB signaling in NSCs. We used the Cor-1 cells as a model system to explore this as well as some other aspects of eCB signaling. We used micro array analysis to address the question if there is a transcriptional overlap between EGFR, FGFR and/or CB1/2 receptor signaling. A strong overlap would be a reasonable indicator for the EGFR and or FGFR to be upstream the CB receptors. A very high correlation was seen between the used CB1 receptor antagonists as well as the CB2 receptor antagonists and furthermore with each other (Figure 5.1). This first of all is proof of principle confirmation that we were able to compare receptor signaling efficiently with each other and moreover shows that CB1 and CB2 receptors signal via very similar pathways in Cor-1 cells. The comparison between FGFR inhibition and CB1/2 receptor antagonist shows a very small correlation indicating that FGFR is not upstream of the CB receptors in Cor-1 cells while they are proliferating under normal growth conditions. Moreover, while FGF-2 was found to be of some importance for Cor-1 proliferation, cells were able to proliferate in the absence of FGF-2 (Figure 5.2). Together this indicates that it is unlikely that FGFR is upstream of CB1/2 receptor signaling.

A smaller overlap between the transcriptional response of CB1/2 receptor signaling and EGFR receptor was identified (Figure 5.1). Moreover, the EGFR does not appear to be directly upstream of eCB signaling in the context of Cor-1 cell proliferation, as CB1/2 receptor agonism cannot recover Cor-1 cell proliferation in low or no EGF conditions (Figure 5.2 B). While there are several interpretations, one particularly plausible possibility would be a common downstream pathway and the mTOR pathway appeared to be particularly promising (personal communication with Dr Gareth Williams) and was further investigated.

Stem cells can adapt to metabolic fluctuations (Speder et al., 2011) and furthermore the eCB system has a role in energy balance regulation via the CB1 receptor (discussed in Rivera et al., 2011). Moreover brain cell progenitor proliferation has been linked to insulin signaling (Hodge et al., 2004; Popken et al., 2004; Lehtinen et al., 2011) and insulin is also present in the growth media the Cor-1 cells are grown in making insulin signaling an attractive target to investigate in the context of eCB driven proliferation. Since insulin is present in the N2 supplement, as a first step we had to test an "in house" N2 supplement to be able to grow the Cor-1 cells in insulin free media. We compared Cor-1 cell proliferation using the commercial and "in house" N2 supplement and no difference was observed (Figure 5.3 A). Cor-1 cell proliferation is significantly reduced in the absence of insulin (Figure 5.3 D). While CB1+2 receptor agonists treatment restores Cor-1 proliferation in the absence of insulin back to near normal proliferation levels, the proliferation could be further reduced using a CB1+2 receptor antagonist in the absence of insulin. This indicates that insulin signaling is perhaps upstream of CB1/2 receptors, but other signaling pathways have an additional effect, and more direct evidence would be required to test this hypothesis. To further investigate the relationship between insulin signaling and CB1/2 receptor signaling in Cor-1 proliferation we wanted to establish which insulin detecting receptors are expressed by the Cor-1 cells. The IGFR is for example expressed by cortical progenitor cells during development (Lehtinen et al., 2011) while cell proliferation in the adult rodent SGZ is impaired in insulin-deficient and insulin resistant rodents (Stranahan et al., 2008; Ming and Song, 2011). There is some evidence at the protein level that InsR as well as the IGFR are expressed by the Cor-1 cells (Figure 5.4), but while the tested IGFR inhibitor showed no effect on Cor-1 proliferation (Figure 5.5), a promising reduction of Cor-1 cell proliferation was observed using the InsR and IGFR inhibitor AG1024. In this context of Cor-1 proliferation the InsR is a more likely candidate than the IGFR to be involved in this process since the IGFR inhibitor did not show an effect. However further experiments are needed to confirm this.

The MAPK cascade is a very prominent example for the mediation of receptor activation via phosphorylation events (Choi et al., 2008). As previously discussed, there are compelling indications for DAGLα and DAGLβ to be phosphorylated and we wanted to investigate possible kinases carrying out their phosphorylation in the context of Cor-1 proliferation. PKA, PKC, CaM kinase, and CDK 1,2,5 have been identified as the best candidates (personal communication Emma Williams, unpublished observations) to carry out the phosphorylation. Kinase inhibitors against the respective kinases were used in a Cor-1 proliferation experiment and all lead to an inhibition of proliferation (Figure 5.7). Treatment with CB1/2 receptor agonists did not recover Cor-1 proliferation in the presence of the CaM kinase or CDK1,2,5 indicating that the inhibited phosphorylation events are not upstream of CB1/2 receptors and therefore unlikely to be DAGL related. The reduction of the PKA inhibitor treatment at least partially was elevated by CB1/2 receptor agonist treatment but this however was not a significant elevation in the tested conditions. Treatment with the PKC inhibitor led to a change in Cor-1 morphology and/or proliferation, which could be significantly recovered by CB1/2 receptor agonist treatment. Further experiments are needed to establish if this is due to the recovery of proliferation or changed morphology, however in either case the results indicated that a phosphorylation event upstream of the CB1/2 receptors is involved in the change. It has been reported that PKA and PKC are involved in 2-AG level control possibly via the DAGLs (Vellani et al., 2008) and an effect via PKC phosphorylation on DAGL activity has been described elsewhere (Perez Roque et al., 1998). Moreover PKC has been demonstrated to affect other components of the eCB system by phosphorylating the CB1 receptor and thereby disrupting its activation (Garcia et al., 1998). Taken together, these results highlight that PKC in particular is likely to be involved in DAGL phosphorylation.

As mentioned above, we hypothesize that the mTOR pathway is a common downstream pathway of the EGFR and CB1/2 receptors. As a first step we wanted to establish the effect of the mTOR inhibitor rapamycin on Cor-1 proliferation and observed a rapamycin mediated reduction in Cor-1 proliferation (Figure 5.8) and thereby establishing the relevance of mTOR pathway for Cor-1 proliferation. Next,

we established that mTOR inhibition by rapamycin leads to a loss of phosphorylation of p70 S6 kinase (Figure 5.9). While not being the only downstream target of mTOR, the p70 S6 kinase has been demonstrated to be important for cell proliferation and therefore was chosen for this investigation (Wing et al., 2005; Varma and Khandelwal, 2007). Cor-1 cells were treated with either EGFR, FGFR (Figure 5.10) or CB1 and/ or CB2 receptor inhibitors (Figure 5.11) and the effect on the phosphorylation status of p70 S6 kinase was observed. More precisely the phosphorylation of threonine 389 which is closely linked to p70 S6 kinase activation (Weng et al., 1998) was investigated using western blot analysis. Treatment with the EGFR inhibitor led to a loss of phospho p70 S6 kinase, while not changing overall p70 S6 kinase levels. This goes hand in hand with the observation that EGF or insulin treatment led to phosphorylation of p70 S6 kinase (Galbaugh et al., 2006). FGFR inhibitor treatment on the other hand had no effect on the phosphorylation status of p70 S6 kinase in our hand, neither had the CB1 and/or CB2 receptor inhibitors. These results indicate that p70 S6 kinase is downstream of EGFR signaling, but not FGFR or CB1/2 receptor signaling and is not the proposed common downstream pathway between EGFR and the CB1/2 receptors.

CHAPTER VI: Results 4

Characterization of DAGL activity in Cor-1 cells

6.1. Introduction

As mentioned in the previous results chapter, there is accumulating evidence, that both DAGLα as well as DAGLβ can be phosphorylated, which is a widespread mechanism to regulate the activity of numerous enzymes (Olsen et al., 2006). Hence it is very likely, that DAGL activity is regulated by phosphorylation, and the sole knowledge about expression is not sufficient to understand DAGL function. Measuring 2-AG levels is far from trivial and a simple "96-well" plate assay for DAGL activity would allow us to investigate what is driving DAGL dependent eCB signaling, which kinases might regulate DAGL activity by phosphorylation of and many more DAGL related questions. There recently has been a DAGL activity assay published that utilises two independent surrogate substrates (Pedicord et al., 2011). The fluorogenic substrate is called 6, 8-difluoro-4-methylumbelliferyl-octanoate (DiFMU), while the chromogenic substrate is called 4-nitrophenol butyrate (PNP). The paper describes how DAGL assays were performed on membranes prepared from parental and DAGLα transfected HEK293 cells, and I have modified this assay for use with Cor-1 cells. Both substrates were tested for their suitability for membrane and whole cell assays and it was confirmed that the expression of different DAGL constructs in Cor-1 cell lines leads to an increased DAGL activity. Furthermore, this assay was used to establish if the use of EGFR inhibitors, FGFR inhibitors, or insulin withdrawal has the ability to change DAGL activity.

6.2. Optimization of a membrane based DAGL activity assay using the surrogate substrate PNP

As a first step we had to establish that our membrane preparation method enriches DAGL. To do so membranes were prepared from DAGLα-V5 overexpressing V5α7 cells and used for western blot analysis alongside cell lysates at equal protein concentrations.

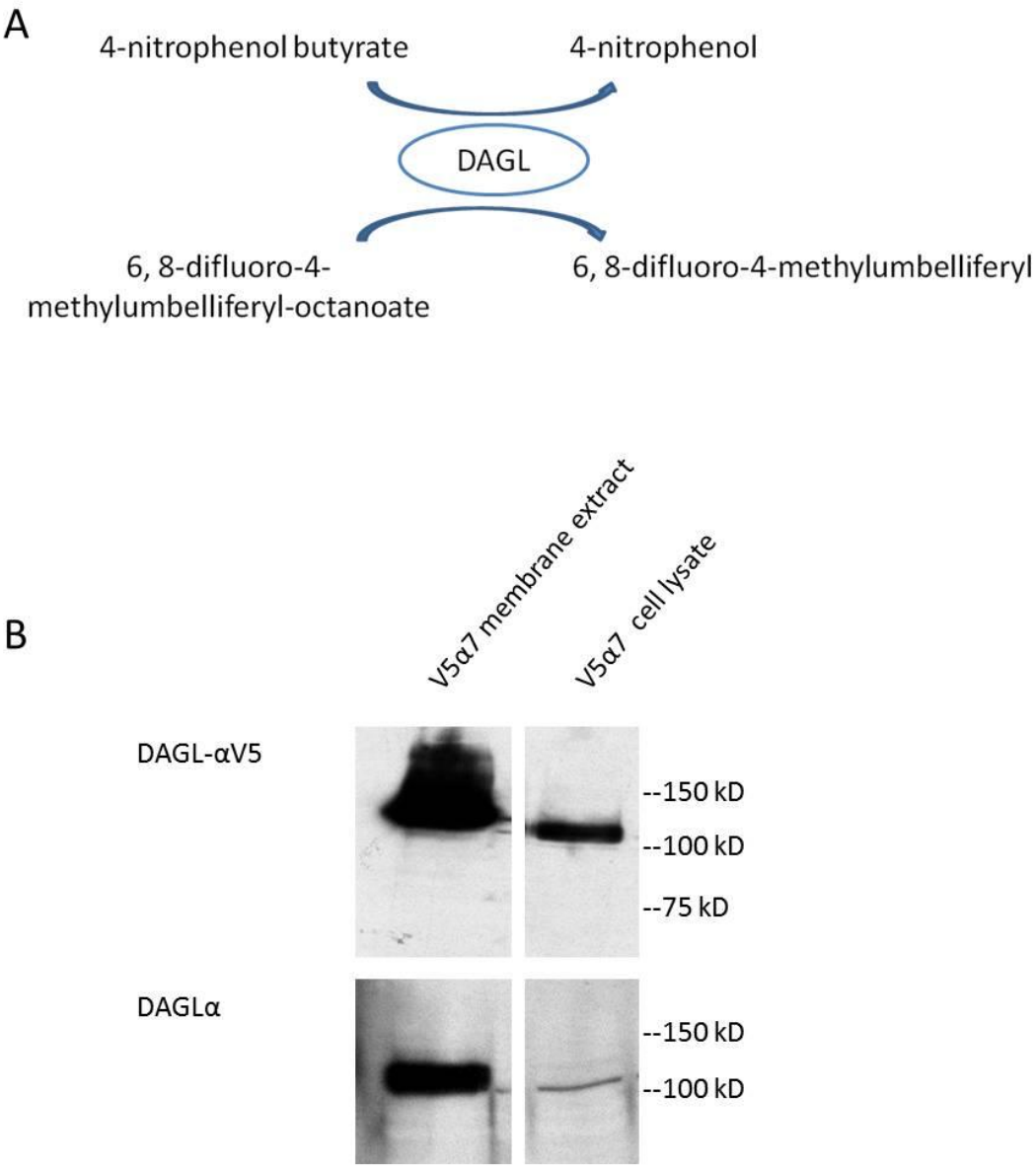


Figure 6.1: DAGLα is enriched in the membrane of V5α7 cells
Reactions of the two surrogate substrates DiFMU and PNP when metabolise by DAGL (A). Membrane extracts or whole cell lysates from V5α7 cells were used for western blot analysis at equal protein concentrations. An anti-V5 antibody was used in A to detect DAGLα-V5 and an anti-DAGLα antibody was used in B.

An antibody to the V5 epitope tag and an antibody against endogenous DAGLα were used to establish the enrichment of DAGLα in the cell membrane fractions. An example of a western blot is shown in Figure 6.1. V5α7 cells express the DAGL-V5 tagged enzyme at 120kDa and furthermore it is substantially enriched in the membrane fraction. An endogenous DAGLα antibody was used to confirm this result (Figure 6.1.B).

After confirming that DAGLα is enriched in the membrane preparation of the V5α7 cells, the membranes were used for DAGL activity assays. The assays were carried out in 96-well clear polypropylene plates, buffers appropriate for the respective substrates were used and the reaction was followed kinetically using the SpectramaxPlus set at a wavelength of 400nm for the substrate PNP and the Flexstation using an excitatory wavelength of 360nm and an emission of 450nm for the DiFMU substrate. The reaction rate was calculated based on the linear regression of the initial 10min using the SoftMaxPro software and typically the mean of 3-4 wells is presented.

A time course using the membranes from Cor-1 or V5α7 cells at a final assay concentration (FAC) of 12.5µg/ml and a substrate concentration of 250µM PNP is shown in Figure 6.2, for control membranes and membranes treated with 1µM THL. The absorbance at 400nm was measured every 12sec for 10min as has been done for all of the following experiments in this section and the mean of three wells is presented. The reaction rate seen for V5α7 membranes is considerably higher than that seen with membranes from parental Cor-1 cells. Moreover, this enhanced activity was completed suppressed by THL (Fig 6.2 A). Use of THL reveals the background activity, perhaps driven by other lipases and other enzymes, and when this signal was subtracted the enhanced activity in the cells that overexpress DAGL was quite considerably higher compared to the parental Cor-1 membranes (Fig 6.2B).

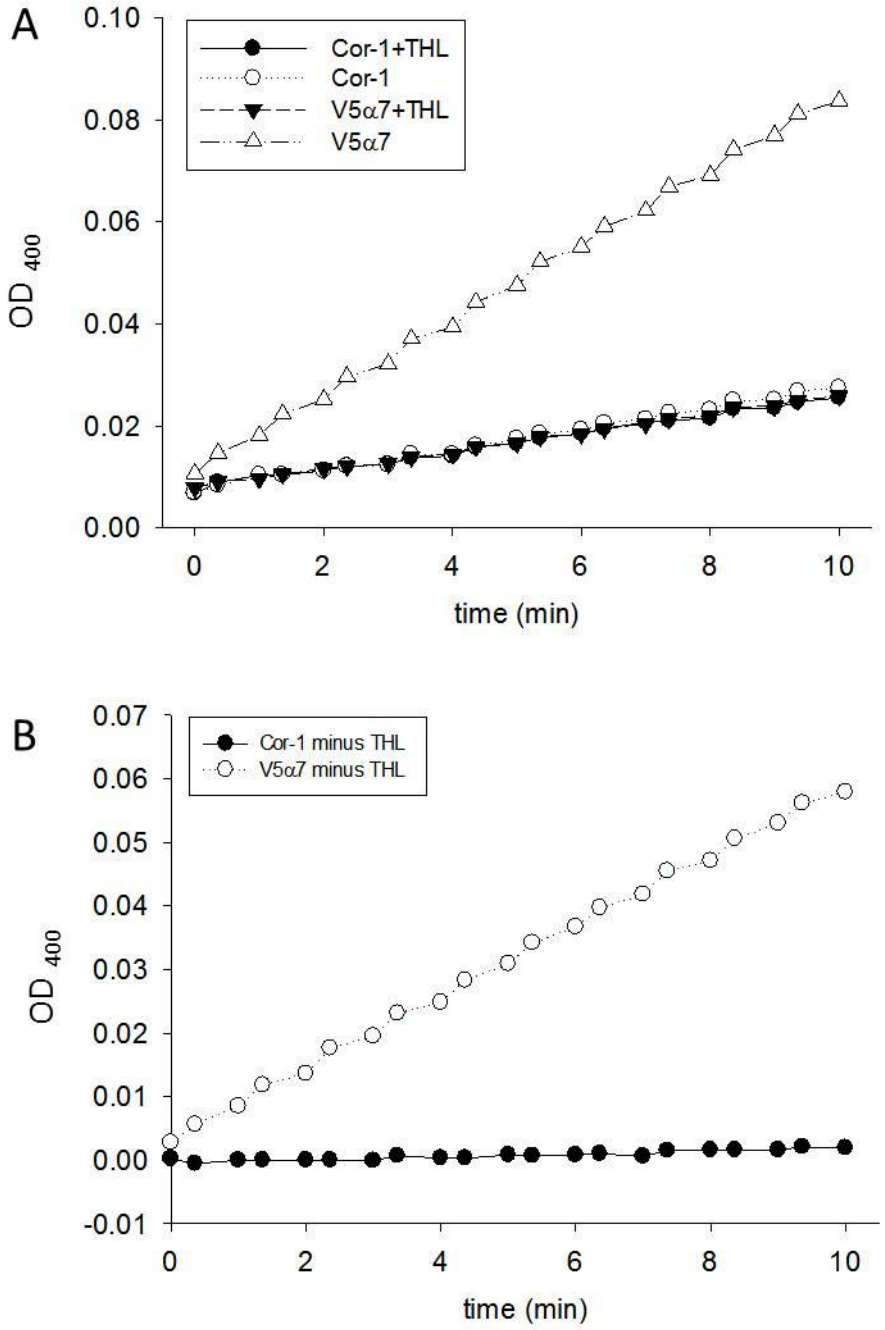
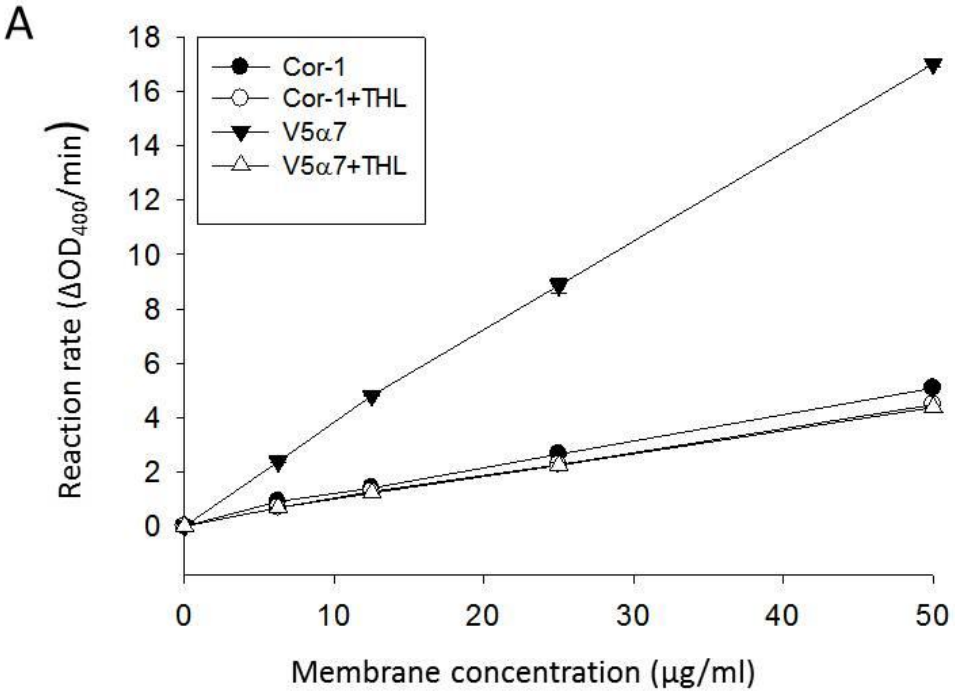
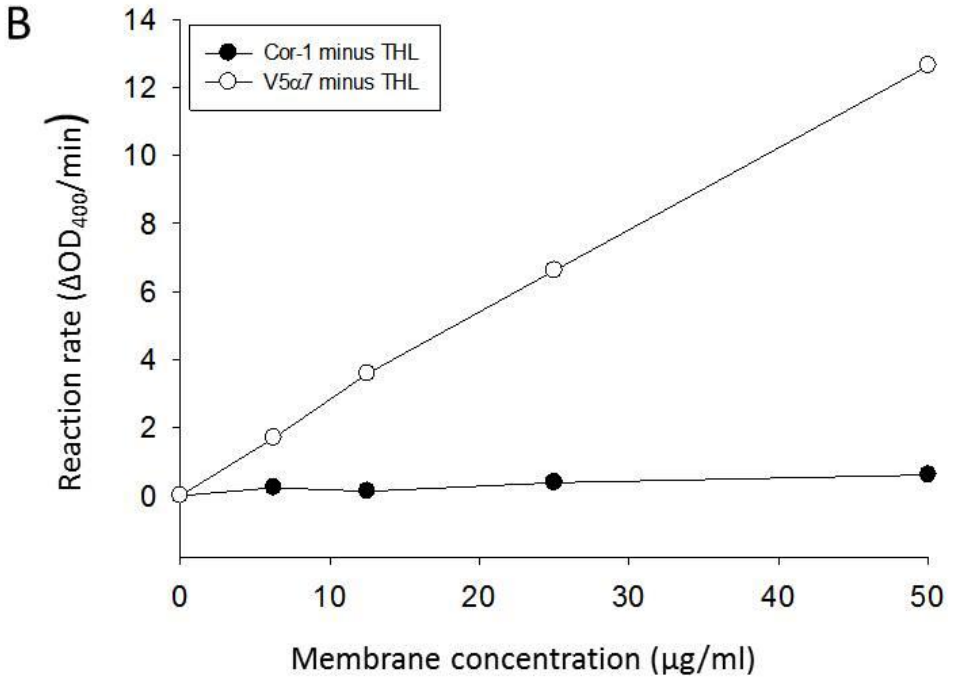


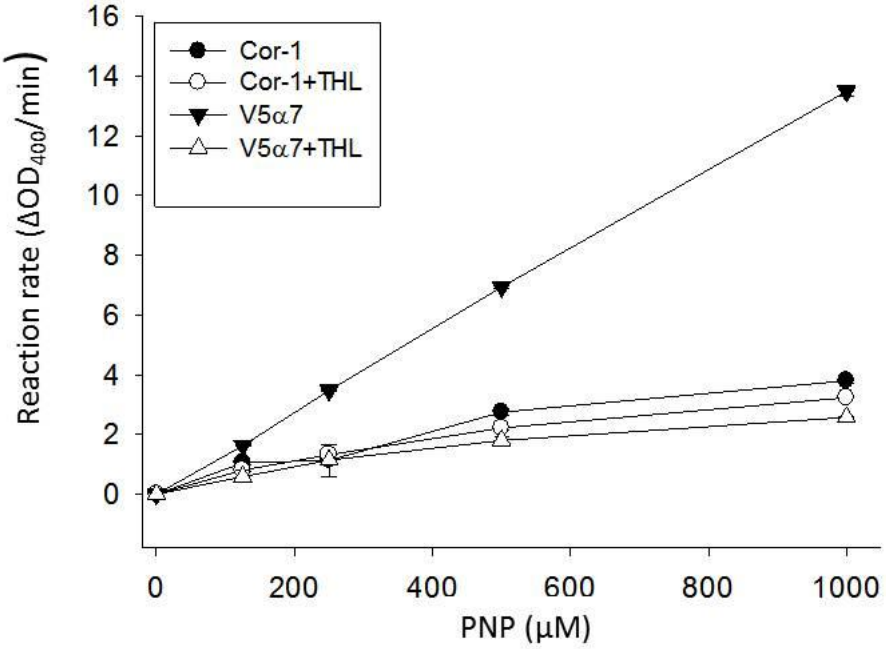
Figure 6.2: Time course for Cor-1 and V5α7 (± THL) absorbance during a DAGLα assay using PNP as the surrogate substrate

Cor-1 and V5α7 membranes were used at 12.5µg/ml FAC and PNP was used as a substrate at a concentration of 250µM. The absorbance was read at 400nm for 10min every 12sec. The mean of three wells is presented (A). The absorbance reading measured for Cor-1/V5α7 membranes in the presence of THL was considered as background and was subtracted from the absorbance signal measured for the untreated membranes (B).


A
Cor-1
Cor-1+THL
V5α7
V5α7+THL
Reaction rate (ΔOD400/min)
Membrane concentration (µg/ml)
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
50


B
Cor-1 minus THL
V5α7 minus THL
Reaction rate (ΔOD400/min)
Membrane concentration (µg/ml)
0
2
4
6
8
10
12
14
0
10
20
30
40
50

C


Cor-1
Cor-1+THL
V5α7
V5α7+THL
Reaction rate (ΔOD400/min)
PNP (μM)
0
2
4
6
8
10
12
14
16
0
200
400
600
800
1000

D

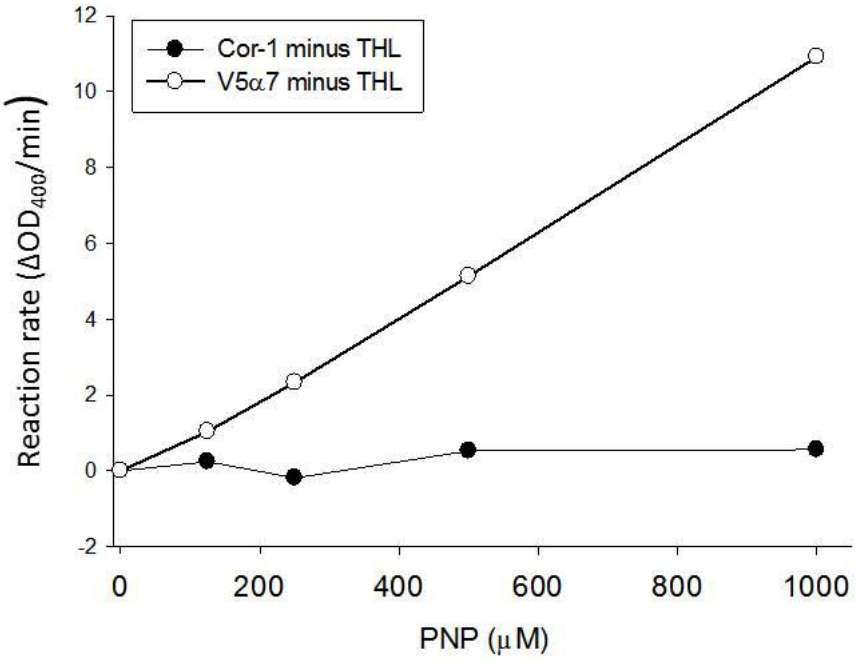

Cor-1 minus THL
V5α7 minus THL
Reaction rate (ΔOD400/min)
PNP (μM)
-2
0
2
4
6
8
10
12
0
200
400
600
800
1000

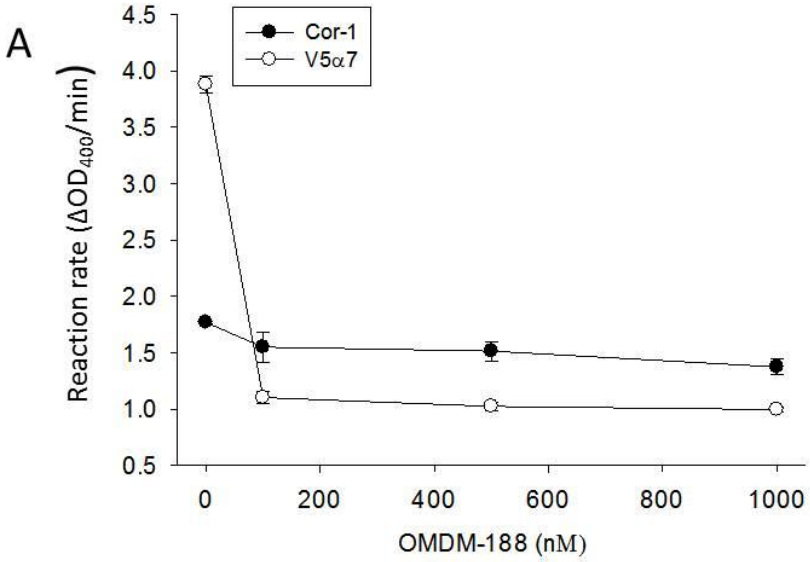
Figure 6.3: Optimization of membrane and PNP substrate concentration for a DAGL assay

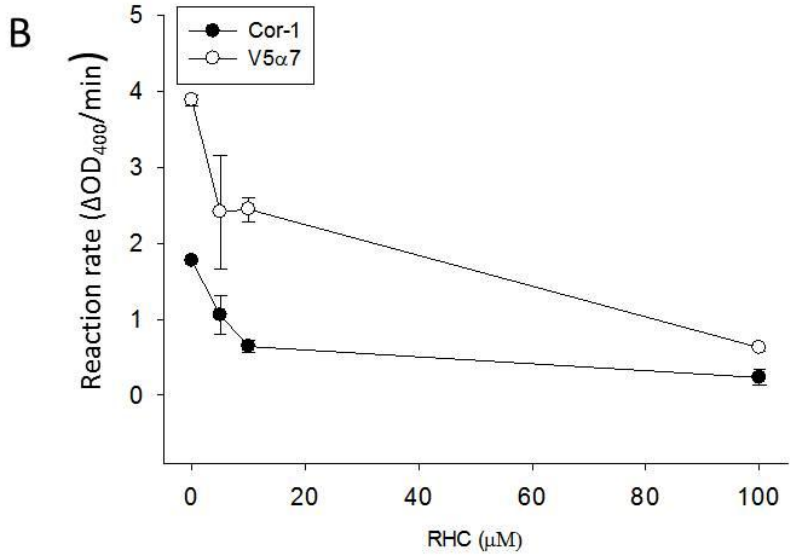
Membranes from Cor-1 and V5α7 cells were extracted and plated at 50, 25, 12.5 or 6.25µg/ml FAC. Half of the membranes were treated with 1µM THL, PNP was used at a concentration of 250µM and absorbance was read at 400nm for 10min (A+B). Membranes from Cor-1 and V5α7 cells were extracted and plated at 12.5µg/ml and treated with different substrate concentrations ranging from 0-1000 µM PNP. Half of the cells were treated with 1µM THL. The absorbance was read at 400nm for 10min every 12sec (C+D). The THL insensitive background signal was subtracted in B and D. The mean of three wells ± SEM is presented.

The effect of varying the membrane concentrations in the PNP DAGL activity assay were established and presented in Figure 6.3 A and B. Cor-1 and V5α7 membranes were used at a range between 50 and 6.25μg/ml FAC ±1μM THL at a PNP concentration of 250μM. The THL sensitivity (presumably DAGLα activity) was only seen with membranes isolated from the V5α7 cells, and not with parental cells. This further supports the conclusion that the activity is indeed dependent on the transgene and suggests that DAGL activity is below a readily detectable value in the parental cells. A membrane concentration of 12.5μg/ml was chosen for all further experiments. At this membrane concentration the reaction rate of the V5α7 membranes is approximately 4 times higher compared to the parental Cor-1 membranes, but a bigger difference can be seen at higher membrane concentrations.

We next varied the substrate concentration (Fig 6.2 C and D). Again, the reaction rate was linearly related to substrate concentration with membranes from V5α7 cells, with no such relationship seen with membranes from parental cells when the THL background was subtracted. Based on the experiments above, it was decided to do all further experiments using a PNP substrate concentration of 250μM unless otherwise stated, as it is well within the linear range of the assay. Even though Cor-1 cells express both DAGL isoforms (Goncalves et al., 2008), the endogenous activity levels in Cor-1 cells are relatively small and are below the detection ability of this novel DAGL assay, at least in these initial experiments. The proportion of the reaction rate seen for V5α7 membranes, most likely is down to the transgenic DAGLα activity.

We next tested the effects of three DAGL inhibitors on the activity measured in the membranes of the parental and transfected Cor-1 cells (Figure 6.4). OMDM-188 is the most potent DAGL inhibitor described to date (Ortar et al., 2008), and this fully inhibited the activity measure in the membranes from the DAGLα transfected V5α7 cells at 100nM. Again, this compound only had a marginal effect on the much lower activity measured with membranes from parental cells (Fig 6.4 A).


A
Cor-1
V5α7
Reaction rate (ΔOD400/min)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
200
400
600
800
1000
OMDM-188 (nM)


B
Cor-1
V5α7
Reaction rate (ΔOD400/min)
5
4
3
2
1
0
0
20
40
60
80
100
RHC (μM)

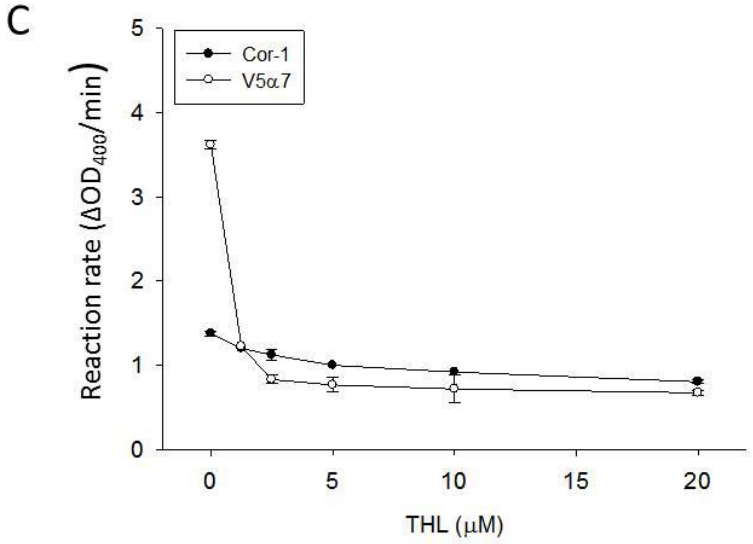

C
Cor-1
V5α7
Reaction rate (ΔOD400/min)
5
4
3
2
1
0
0
5
10
15
20
THL (μM)

Figure 6.4: Inhibiting DAGL activity by using the DAGL inhibitors THL, RHC and OMDM-188

Membranes from Cor-1 and V5α7 cells were extracted and plated at 12.5µg/ml FAC. A substrate concentration of 250µm PNP was used and the absorbance is read at 400nm for 10min every 12sec. Three different DAGL inhibitors were used to establish their effect on DAGL activity in this assay. OMDM-188 was used at concentrations of 0, 100, 500 and 1000nM (A), RHC was used at concentrations of 0, 5, 10 and 100µM (B) and THL was used at concentrations of 0, 1.25, 2.5, 5, 10, 20 µM. The bars show the mean of three wells ± SEM.

RHC80267 inhibited activity in membranes from the DAGL transfected cells in a dose-dependent manner, with a ~50% inhibition seen at 10μM and an almost complete inhibition at 100 μM (Fig 6.4 B). THL was seen to inhibit the differential activity seen between membranes obtained from the DAGL transfected cells compared to the parental cells (Fig 6C). Thus three independent DAGL inhibitors reduced the activity seen in membranes from DAGL transfected cells back to the level seen with membranes from parental cells. These results further support the conclusion that the assay is measuring the activity of the transfected DAGL, and confirm relatively low levels of this activity in the parental cells.

6.3. Optimization of a membrane based DAGL activity assay using the substrate DiFMU

After establishing PNP as a suitable substrate for Cor-1 membrane assays, we tested the fluorogenic substrate DiFMU. A time course using membranes at 12.5μg/ml and a substrate concentration at 10μM DiFMU is shown in Figure 6.5. Using this substrate, the difference between membranes from the parental and DAGL transfected cells was relatively small, and the signal was only partially inhibited by THL (Figure 6.5). Attempts to "tease-out" a better differential signal between the cells by varying membrane concentration (Fig 6.6 A and B) or substrate concentration (Fig 6.6 C and D) showed the best differential to be obtained with membranes at 12.5μg/ml and substrate at 10μM. The highest difference was ~ 2-fold increase between Cor-1 and V5α7 membranes when the THL background was subtracted. Again three DAGL inhibitors were tested and confirmed that the signal can be inhibited by either of them (data not shown) however, results with the non-fluorogenic substrate were clearly better providing a better signal window due to the lower background.

6.4. Whole cell DAGL activity assay

We next wanted to determine if the DAGL assays could be adapted for use with on a whole cells assay. A whole cell assay would allow the user to observe the effect of growth factors or kinase inhibitors on DAGL activity in a whole cell context.

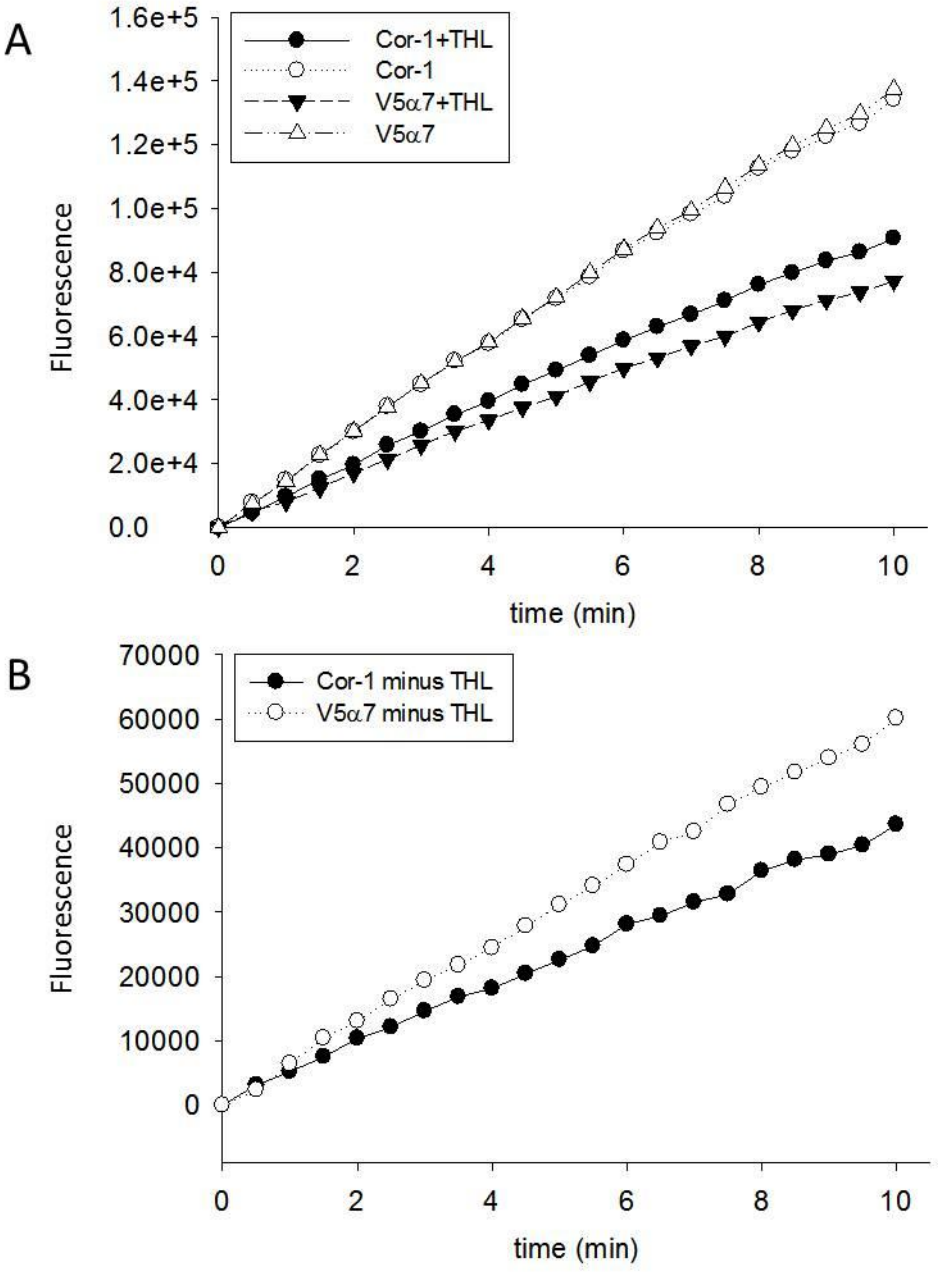
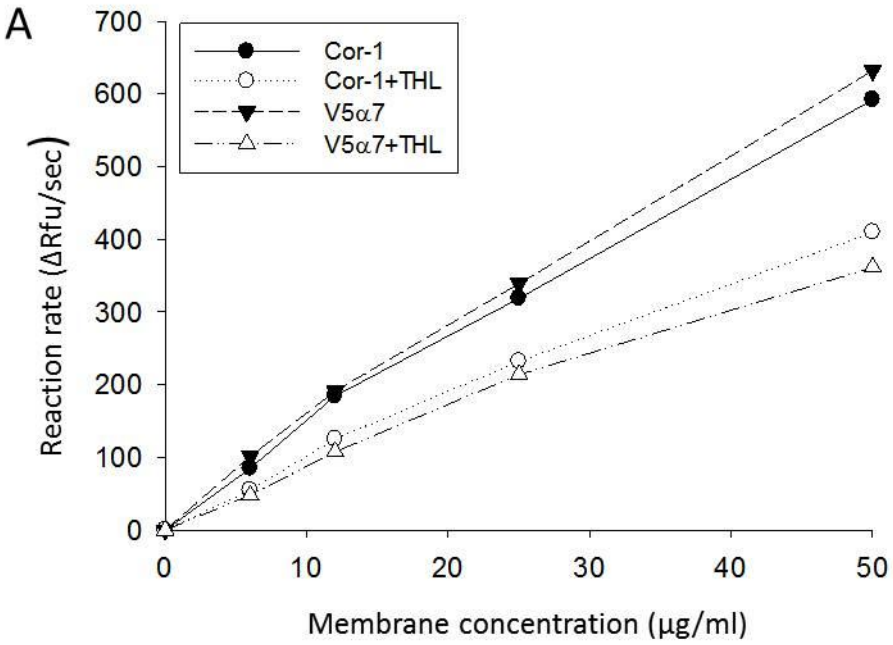
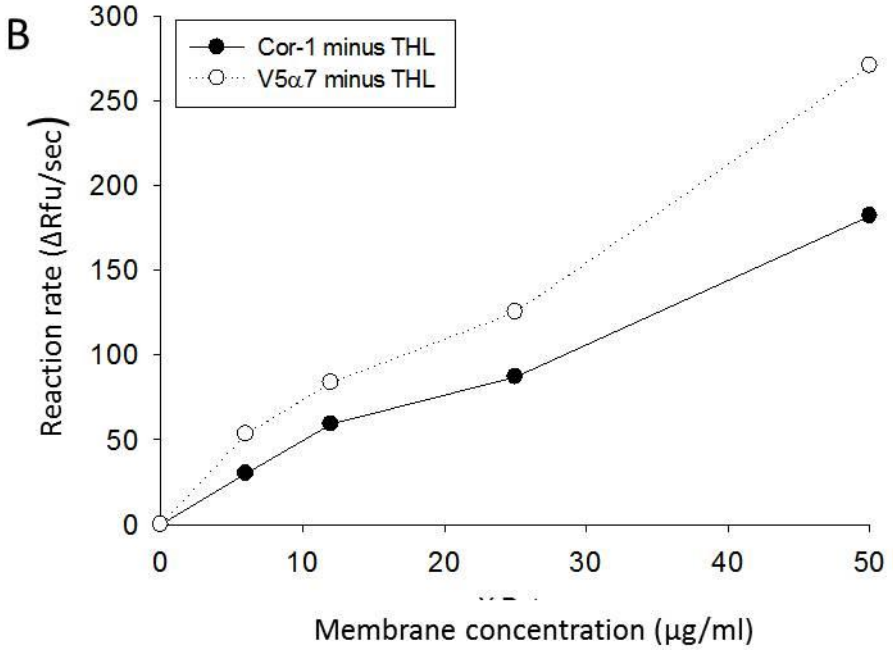


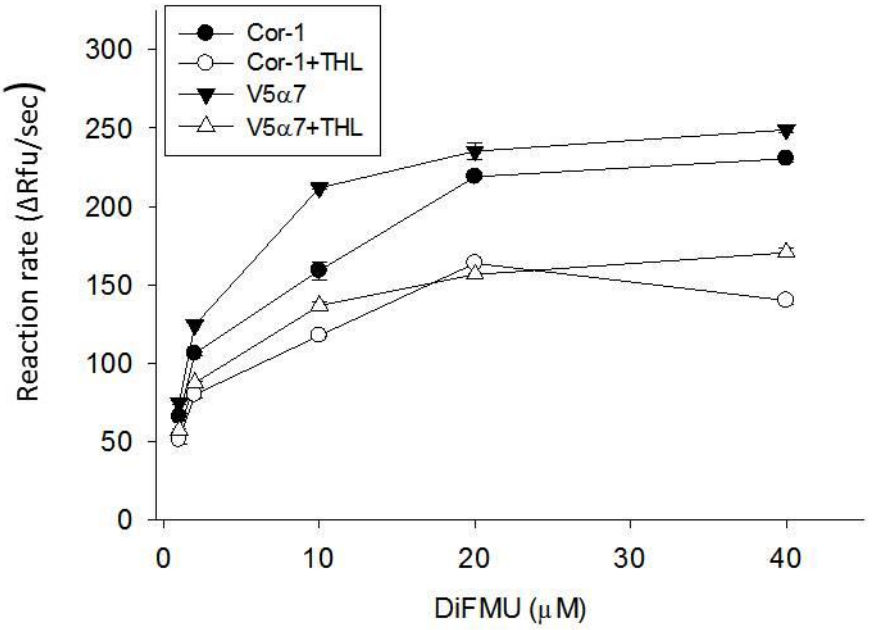
Figure 6.5: Time course for Cor-1 and V5α7 (± THL) fluorescence during a DAGL assay using DiFMU as the surrogate substrate

Cor-1 and V5α7 membranes were used at 12.5µg/ml FAC. The surrogate substrate DiFMU was used at a concentration of 10µM and fluorescence was measured using an excitatory wavelength of 360nm and an emission of 450nm for 10min every 12sec (A). The fluorescence reading measured for Cor-1/V5α7 membranes in the presence of 1µM THL was considered as background and was subtracted from the fluorescence signal measured for the untreated membranes (B). Each time point is the mean of three wells ± SEM


A
Cor-1
Cor-1+THL
V5α7
V5α7+THL
Reaction rate (ΔRfu/sec)
Membrane concentration (µg/ml)
0
100
200
300
400
500
600
700
0
10
20
30
40
50


B
Cor-1 minus THL
V5α7 minus THL
Reaction rate (ΔRfu/sec)
Membrane concentration (µg/ml)
0
50
100
150
200
250
300
0
10
20
30
40
50

C


Cor-1
Cor-1+THL
V5α7
V5α7+THL
Reaction rate (ΔRfu/sec)
300
250
200
150
100
50
0
0
10
20
30
40
DiFMU (μM)

D

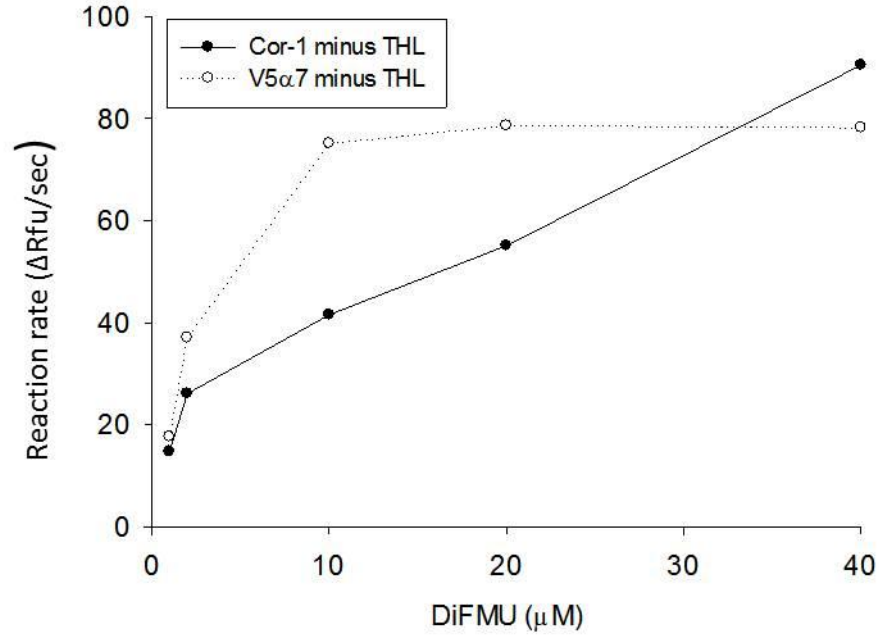

Cor-1 minus THL
V5α7 minus THL
Reaction rate (ΔRfu/sec)
100
80
60
40
20
0
0
10
20
30
40
DiFMU (μM)

Figure 6.6: Optimization of membrane and substrate concentration for a DAGL assay using the substrate DiFMU

Membranes from Cor-1 and V5α7 cells were extracted and plated at 50, 25, 12.5 or 6.25µg/ml FAC. Half of the membranes were treated with 1µM THL, DiFMU was used at a concentration of 10µM and fluorescence was measured using an excitatory wavelength of 360nm and an emission of 450nm for 10 min every 12 seconds (A). Membranes from Cor-1 and V5α7 cells were extracted and plated at a FAC of 12.5µg/ml and treated with different substrate concentrations of 1 ,2 ,10, 20 and 40µM. Half of the cells were treated with 1µM THL and fluorescence was measured using an excitatory wavelength of 360nm and an emission of 450nm for 10min every 12sec (C). The fluorescence reading measured for Cor-1/V5α7 membranes in the presence of THL was considered as background and was subtracted from the fluorescence signal measured for the untreated membranes (B+D). The mean of 3 wells ± SEM is presented.

Cor-1 cells and V5α7 cells were seeded into 96-well plates at a cell density of 40,000 cells/ well and left to attach overnight in growth media. This cell number was chosen based on past experience as it gives ~60-70% cell confluence the next day. To conduct the assay, the growth media was carefully replaced with the appropriate assay buffer and the surrogate substrates PNP and DiFMU were tested on the whole cells.

6.4.1. Optimization of a whole cell DAGL activity assay using the substrate PNP

A substrate range of 0-500µM PNP was tested in this whole cell assay as can be seen in Figure 6.7. Half of the Cor-1 and V5α7 cells were treated with 25µM THL and absorbance was read at 400nm for 10min. The reaction rates increased with increasing substrate concentration, while THL treatment led to a decrease of reaction rate in both cell lines (Figure 6.7 A). The reaction rate in the presence of THL was subtracted from the reaction rate seen for the cells alone, which separates the reaction rates of Cor-1 and V5α7 cells and at 250µM the reaction rate of V5α7 cells was about double the reaction rate seen for Cor-1 cells (Figure 6.7 B). The reaction rates for the DAGL transfected cells increased linearly with substrate concentration, whereas the reaction rate seen with parental cells did not differ between 50 and 500 µM PNPB. The DAGL transfected cells hydrolysed the substrate 2-3 times better than the parental cells at higher substrate concentrations, suggesting that this assay can be used to get an index of DAGL activity in living cells. Again, the difference between the DAGL transfected and parental cells was abolished by treatment with THL, RHC80267 and OMDM-188 (data not shown). However, the high basal rate of substrate hydrolysis seen with parental cells was only partially inhibited by the DAGL inhibitors, and this would be a confounding factor in the analysis of data obtained with living cells.

Given the 4 fold window between reaction rates of Cor-1 and V5α7 membranes in the membrane based assay, we expected a similar window for the whole cell assay. This was not the case, even though a better separation can be achieved by subtracting the respective THL backgrounds. The higher background in cell based assays might be caused by cytosolic enzymes.

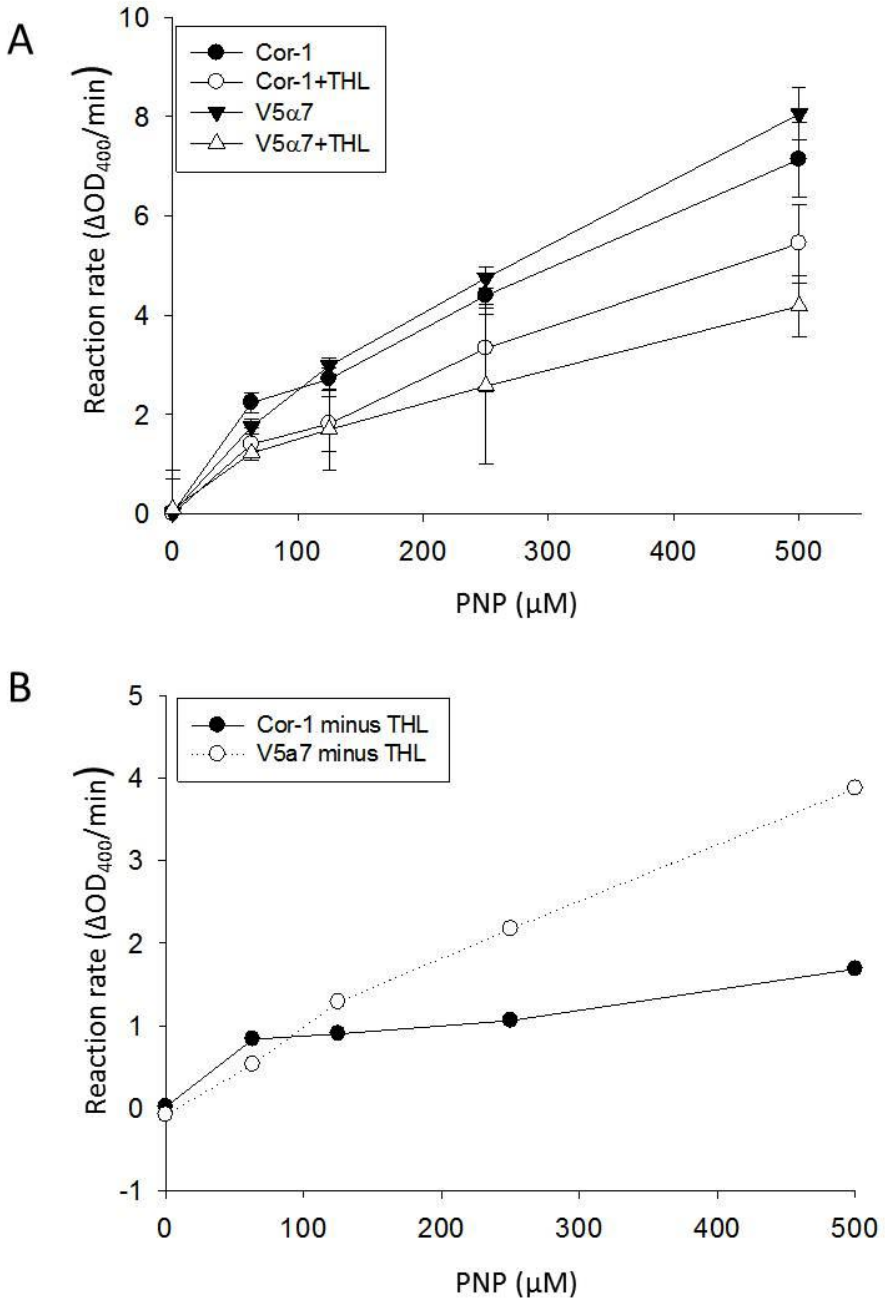


Figure 6.7: Optimization of the PNP substrate concentration for a DAGL whole cell assay

Cor-1 and V5α7 cells were seeded at 40,000 cells/well into a 96-well plate and left to attach overnight before the assay was carried out. Half of the cells were treated with 25μM THL and absorbance was read at 400nm for 10min (A). The absorbance reading measured for Cor-1/V5α7 cells in the presence of THL was considered as background and was subtracted from the absorbance signal measured for the untreated cells (B). The mean of 4 wells ± SEM is presented.

6.4.2. Optimization of a whole cell DAGL activity assay using the substrate DiFMU

Next we wanted to establish the suitability of the fluorogenic substrate DiFMU in a whole cell assay. Cor-1 cells and V5α7 cells were seeded at a cell density of 40,000 cells/well and left to attach overnight in growth media. A substrate range of 0-40µM DiFMU was tested on the whole cell assay as can be seen in Figure 6.8. Half of the Cor-1 and V5α7 cells were treated with 25µM THL and fluorescence is measured using an excitatory wavelength of 360nm and an emission of 450nm for 10min every 10sec. The reaction rates were overall proportional to the substrate concentration, while THL treatment led to a decrease of reaction rate in both cell lines (Figure 6.8). With this substrate the parental cells showed a slightly higher reaction rate compared to V5α7 cells suggesting it is driven by other enzymes, most likely other lipases.

Overall, neither PNP nor DiFMU based whole cell assay were very robust and the window between reaction rates of Cor-1 cells and V5α7 cells was smaller compared to the membrane based assays. We therefore decided to do further experiments based on the membrane assays.

6.5. Applying a membrane based DAGL activity assay to compare differences in DAGL activity

6.5.1. Analysing DAGL activity in different Cor-1 cell lines

Different Cor-1 cell based cell lines were established as mentioned in previous chapters. In brief, V5α7 cells express DAGLα-V5, Mα8 cell express DAGL-V5 with a mutation in a putative d-box and V5β5 cells express DAGLβ-V5. The membrane based DAGL assay was chosen to identify, if the overexpressed enzymes are functional and by how much they increase the measured reaction rates. Membranes from Cor-1, V5α7, Mα8, and V5β5 cells were extracted and plated at 12.5µg/ml FAC onto a 96-well plate. Half of the membranes were treated with 1µM THL and 250µM PNP was used as a substrate for the performed DAGL activity assay (Figure 6.9 A+B). The absorbance at 400nm was read, the reaction rate was normalised to the reaction rate of Cor-1 membranes and data from three independent experiments was pooled.

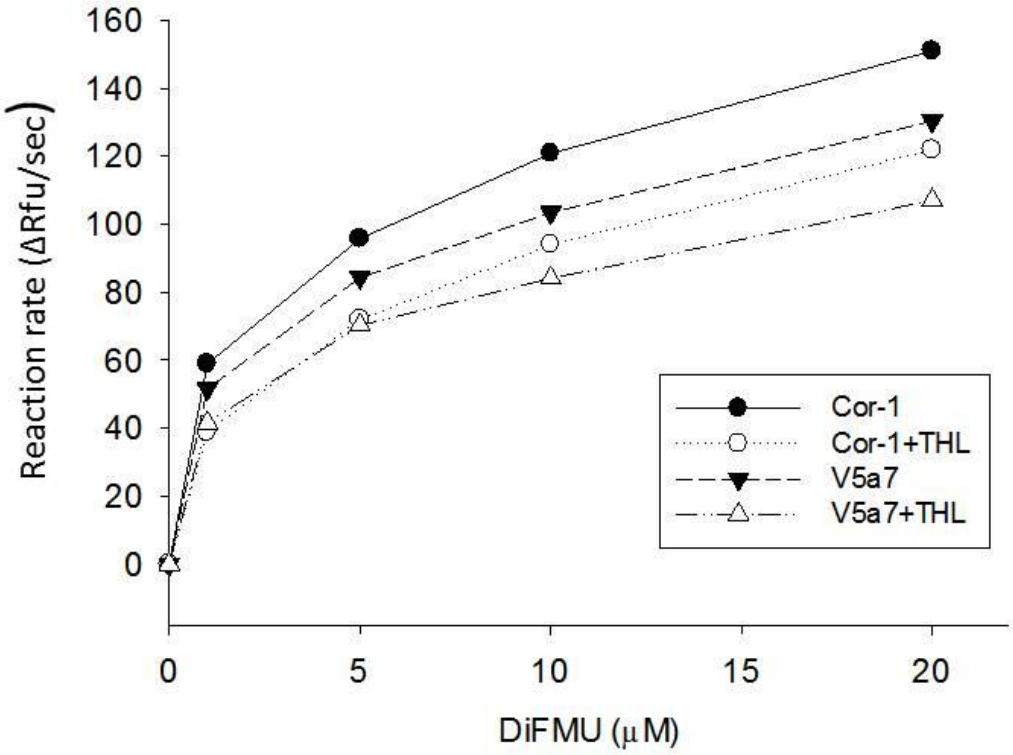


Figure 6.8: Optimization of the DiFMU substrate concentration for a DAGL whole cell assay

Cor-1 and V5α7 cells were seeded at 40,000 cells/well into a 96-well plate and left to attach overnight before the assay was carried out. DiFMU concentrations of 1, 5, 10 and 20µM were used and half of the cells were treated with 25µM THL and the fluorescence was measured using an excitatory wavelength of 360nm and an emission of 450nm for 10min every 12sec. The mean of 4 wells ± SEM is presented.

The relative reaction rates were increased for all the tested membranes from the overexpressing cell lines if compared to the native Cor-1 membranes (Figure 6.9 A). The reaction rate in the presence of THL was defined as background and subtracted from the signal of the uninhibited membranes (Figure 6.9 B). The relative reaction rate of the overexpressing cell line membranes is 4-5 times higher than the relative reaction rate of Cor-1 membranes. This confirms that all three overexpressing cell lines express functional DAGL constructs and all membranes reacted to THL treatment with a reduced reaction rate. It furthermore confirms that DAGLα, as well as DAGLβ can use PNP as a surrogate substrate and that the d-box mutation in Mα8 cells does not abolish DAGL activity.

The same assay design as described above was used for an assay using DiFMU as a surrogate substrate. The fluorescence is measured using an excitatory wavelength of 360nm and an emission of 450nm for 10min, the reaction rate was normalised to the Cor-1 reaction rate and data from three different experiments were pooled (Figure 6.9 C+D). The THL background was subtracted from the relative reaction rate seen for the membranes on their own and is presented in Figure 6.9 D. The relative reaction rate of V5α7 is approximately doubled in comparison to Cor-1 membranes and only a very slight increase in the relative reaction rate for Mα8 and V5β5 in comparison to Cor-1 membranes was observed. Overall this ties in with previous observations during the optimization of the DiFMU membrane assay where only a small window between the parental and transgenic DAGLα expressing cell line was observed and we therefore decided to focus on PNP based membrane assays to address further questions.

6.5.2. Is DAGL activity regulated by EGFR, FGFR and/or insulin?

As described in previous results chapters, one of our aims is to establish what drives the eCB signaling in Cor-1 cells. The three investigated contenders for this role are EGF, FGF and InsR signaling and we designed a DAGL activity assay aiming to address this question. V5α7 cells were grown in growth media, treated for 4h with 100nM AG1478 and 500nM PD173074 (EGFR/FGFR inhibitors respectively) or grown in the absence of insulin for 48h.

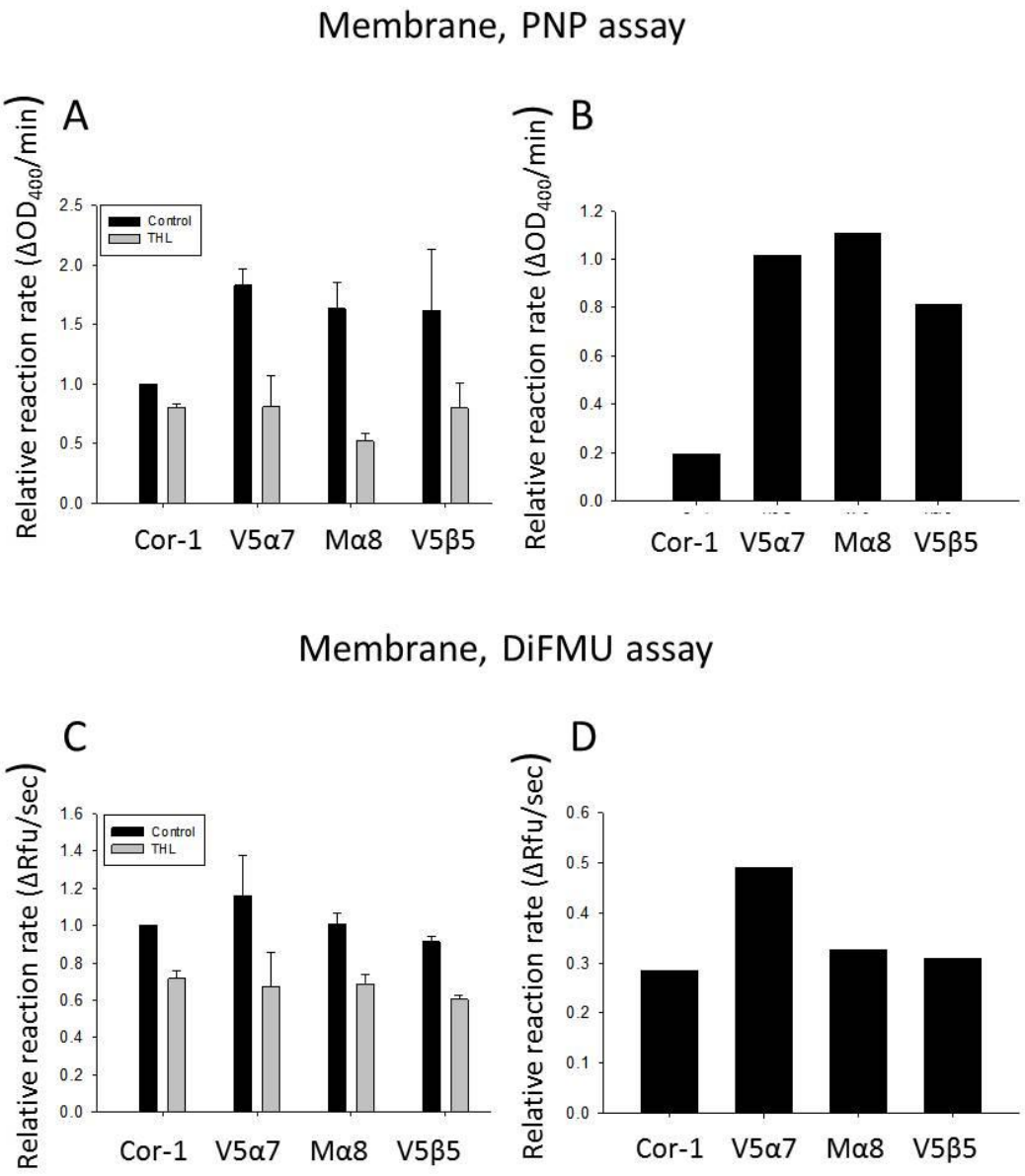
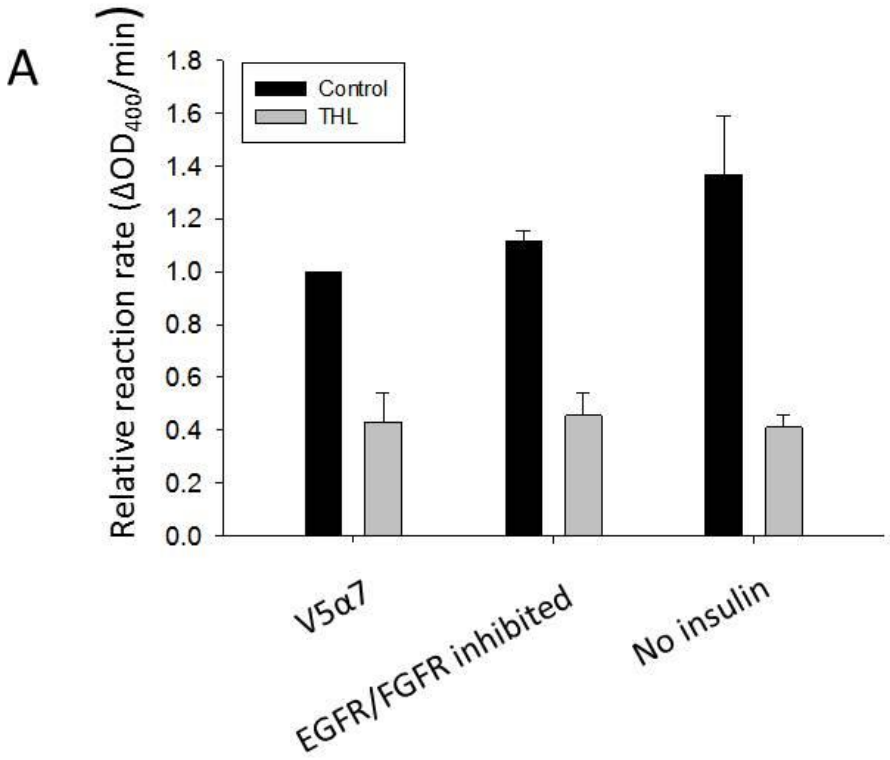

Membrane, PNP assay
A
B
Relative reaction rate (ΔOD400/min)
Control
THL
Cor-1
V5α7
Mα8
V5β5
Membrane, DiFMU assay
C
D
Relative reaction rate (ΔRfu/sec)

Figure 6.9: Comparison between DAGL activity of Cor-1 cells and the cell lines V5α7, Mα8 and V5β5

A membrane based assay was carried out using PNP at a concentration of 250µM. Membranes from Cor-1, V5α7, Mα8 and V5β5 cells were extracted and plated at 12.5µg/ml FAC onto a 96-well plate. Half of the membranes were treated with 1µM THL and the absorbance was read at 400nm for 10min, the reaction rate was normalised to the Cor-1 reaction rate and data from three independent experiments was pooled (A+B). A membrane based assay was carried out using DiFMU as a substrate at the concentration of 10µM. Membranes from Cor-1, V5α7, Mα8 and V5β5 cells were extracted and plated at 12.5µg/ml FAC. Half of the membranes were treated with 1µM THL and the fluorescence was measured using the excitatory wavelength of 360nm and an emission of 450nm. The reaction rate was normalised to the Cor-1 reaction rate and data from three independent experiments was pooled (C+D). The relative reaction rate in the presence of THL was subtracted from the relative reaction rate of the uninhibited membranes (B+D). The relative reaction rate is presented as the mean ± SEM.


A
Relative reaction rate (ΔOD400/min)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
THL
V5α7
EGFR/FGFR inhibited
No insulin

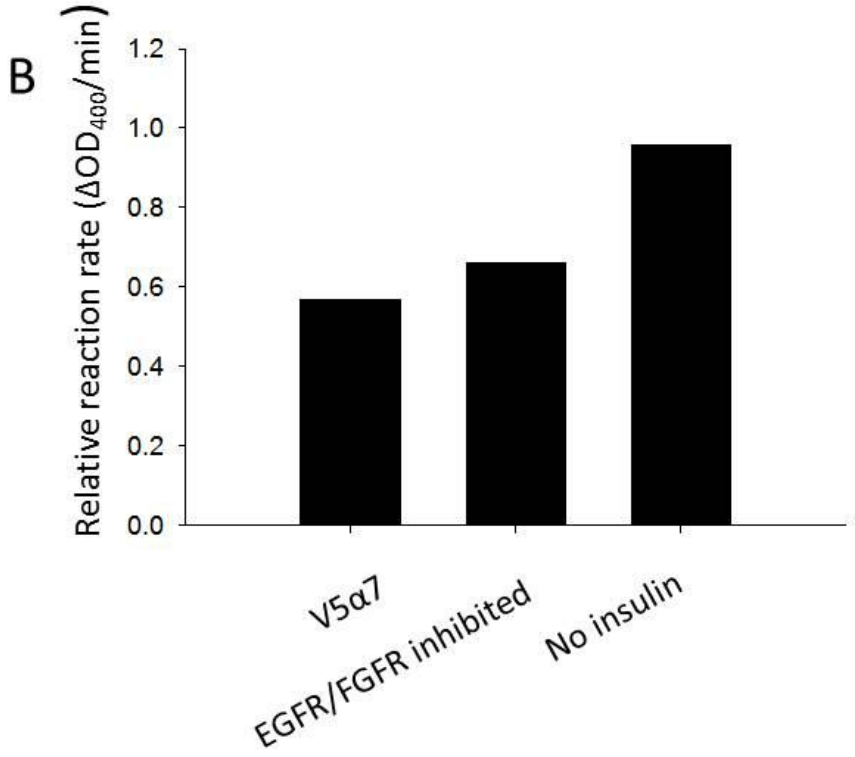

B
Relative reaction rate (ΔOD400/min)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
V5α7
EGFR/FGFR inhibited
No insulin

Figure 6.10: DAGL expression level in V5α7 under control conditions or grown with EFGR+FGFR inhibitors or in the absence of insulin

V5α7 cells were grown in growth media, treated for 4h with 100nM AG1478 and 500nM PD173074 (EGFR/FGFR inhibitors respectively) or grown in the absence of insulin for 48h. The membranes were extracted and plated at 12.5 µg/ml FAC onto a 96-well plate. Half of the membranes were treated with 1µM THL and 250µM PNP was used as a substrate. The absorbance is read at 400nm for 10min and the reaction rate was normalised to the V5α7 membrane reaction rate (A+B). The relative reaction rate in the presence of THL was defined as background and subtracted from the relative reaction rate measured for the membranes on their own (B). The mean of three independent experiments ± SEM is presented.

The membranes from these cells were extracted and plated at 12.5µg/ml FAC onto a 96-well plate. Half of the membranes were treated with 1µM THL and 250µM PNP was used as a substrate. The absorbance is read at 400nm for 10min, the reaction rate was normalised to the V5α7 cell reaction rate and data from three independent experiments was pooled (Figure 6.10 A+B). The relative reaction rate in the presence of THL was defined as background and subtracted from the relative reaction rate of the membranes without THL (Figure 6.10 B). The relative reaction rate is slightly increased for the membranes treated with EGFR/FGFR inhibitors and increased by 25-30% in membranes from the cells grown without insulin. This trend however is not significant, but encouraging to further investigate the role of insulin on DAGL activity.

6.6. Conclusions and discussion

The measurement of the eCBs, 2-AG and anandamide, is challenging and relatively time consuming (Gao et al., 2010; Zoerner et al., 2012) and therefore we wanted to investigate a recently published DAGL assay using surrogate substrates (Pedicord et al., 2011). Here we tested the surrogate substrates PNP and DiFMU in membrane based and whole cell assays using parental and DAGLα transfected Cor-1 cells.

DAGLα is considerably enriched using a membrane extraction method compared to the lysate (Figure 6.1), which is not surprising, as DAGLα is thought to be associated with the membrane. The substrate PNP was tested (Figure 6.2+6.3) on parental Cor-1 membranes and membranes from the transgenic DAGLα expressing cell line. Only the signal of V5α7 membranes can be convincingly inhibited using THL indicating that the DAGLα activity in Cor-1 cells lies below the detection level of this assay. A substrate as well as membrane dependent increase of the reaction rate can be seen for V5α7 membranes and on average, a 4 fold difference was seen between the reaction rate of Cor-1 cells and V5α7 membranes at a FAC of 12.5µg/ml and a PNP concentration of 250µM, which are the most commonly used concentrations in our experiments. A bigger difference was seen at higher membrane and substrate concentrations. OMDM-188, THL, and RHC80267 were tested on Cor-1 and V5α7 membranes (Figure 6.4) and they inhibited the reaction rates. In the case of OMDM-188 and THL the reaction rate of V5α7 membranes fell

below that seen for Cor-1 membranes and this might indicate a slightly different background, which is not inhibited by the DAGL inhibitors in Cor-1 compared to V5α7 membranes as they are a clonal cell line.

Membrane assays using DiFMU as a substrate were carried out and the reaction rates seen for Cor-1 and V5α7 membranes were very similar (Figure 6.5+6.6). Subtracting the "THL background" from the signal, a difference can be seen between the parental and the transgenic cells. However the small difference between the two membrane types imposes limitations for the use of DiFMU in Cor-1 membrane assays (Figure 6.4).

Given the success using PNP as a surrogate substrate in the membrane based assay, we wanted to establish, if PNP can be used in a whole cell assay. This would allow an even quicker mean of screening DAGL activity. While both cells' DAGL activity could be inhibited using THL as well as OMDM-188 and RHC80267 (data not shown), the reaction rate seen for Cor-1 cells was very similar to the reaction rate seen for V5α7 cells under all conditions (Figure 6.7 A). A similar result was seen when DiFMU was used as a substrate in a Cor-1 whole cell assay (Figure 6.8). The overall lack of robustness and a clear window between parental and transgenic cell activity lead to the decision to focus on membrane based assays.

Next, we wanted to establish if the overexpressing cell lines V5α7, Mα8, and V5β5 display an increase in DAGL activity. To do so, we carried out PNP and DiFMU based membrane assays. Using PNP as a substrate, the relative reaction rates of all three cell lines were significantly increased compared to Cor-1 membranes (Figure 6.9 A+B). This indicates, DAGLβ can use PNP as a surrogate substrate while the putative d-box mutation in DAGLα has no fundamental effect on enzyme activity. Using DIFMU as a surrogate substrate to carry out the same set of experiments no significant difference between relative reaction rates was seen for the different Cor-1 cell lines (Figure 6.9 C+D), which is in line with previous observations showing a very limited window between Cor-1 and V5α7 membrane reaction rates while establishing assay conditions (Figure 6.6). Therefore this reflects a limitation of DiFMU based assays in Cor-1 cells rather than being a reflection on the DAGLs

activity status and therefore all further questions were addressed using the PNP based membrane assay.

The EGFR, FGFR, and insulin signaling were explored as possible mechanisms to drive eCB signaling in Cor-1 cells (see previous Chapter). To investigate the direct influence on DAGL activity, V5α7 cells were treated with the EGFR and FGFR inhibitors or deprived of insulin (Figure 6.10). EGFR/FGFR inhibitor treatment did not fundamentally change DAGL activity, consequently underlining previous results in this thesis, that eCB signaling is not influenced significantly by EGF/FGF signaling in Cor-1 cells. Insulin deprivation, however, leads to an increase in DAGL activity (Figure 6.10 B). While not being a significant difference (student t-test), this observation is interesting in combination with our previous results and encourages to further explore the relationship between insulin and DAGL driven eCB signaling.

A DAGL surrogate substrate assay based on commercially available substrates allows cheap and large scale screens to monitor DAGL activity. However it has to be mentioned, that these results always have to be verified using the native substrate. We proposed, that DAGL has a "lid" region, which is potentially capable of limiting substrate access to the catalytic side (Reisenberg et al., 2012). Differences in substrate size or other properties might allow the substrate to be metabolised by DAGL even when the "lid" prevents access to the catalytic side for DAG. The surrogate substrate DiFMU overall failed to be suitable as a surrogate substrate for use on Cor-1 membranes in our hands. However a significant signal window was seen for PNP. It has to be mentioned, that in Cor-1 cells the overexpression of DAGLα as well as DAGLβ leads to an increase in signal when PNP is used as the surrogate substrate, indicating that it can be used as a surrogate substrate by both which might allow to study both DAGLs. Overall, PNP was a reliable surrogate substrate in the membrane based assay and will be utilized for further studies.

CHAPTER VII: Discussion

Since the initial stages of eCB research many discoveries have linked this signaling pathway with a wide range of physiological processes. Our lab is particularly interested in the effect the eCB system has on adult neurogenesis and have used the Cor-1 NSC line in combination with other *in vitro* and *in vivo* methods to establish a correlation between eCB signaling and progenitor cell proliferation (Goncalves et al., 2008). To gain a better understanding of eCB signaling overall it is essential to expand the knowledge about eCB regulation as well as putting it into context with the extended signaling network. DAGLα is at the heart of eCB signaling and little is known about its regulation. It is important to investigate this further, as DAGL synthesizes one of the key eCBs.

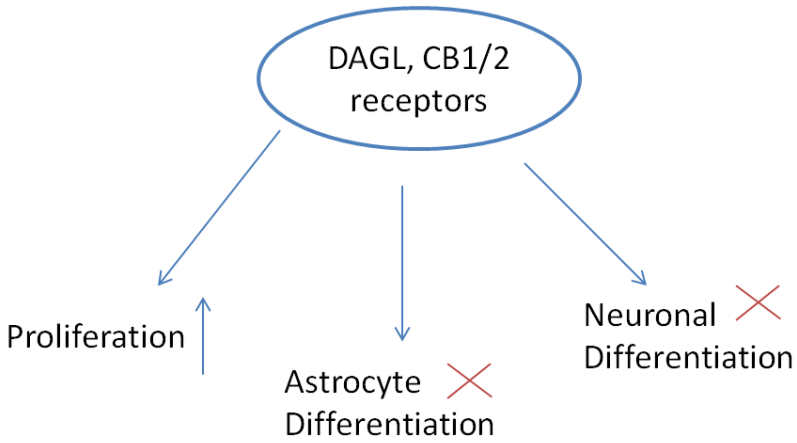


Figure 7.1: Schematic for the role of DAGL and the CB1/2 receptors in proliferation and differentiation

While DAGL as well as CB1/2 receptor signaling is required for the proliferation of neural stem cell proliferation in vitro, no evidence was found indicating that the CB1/2 receptors are needed for differentiation of neural stem cells to astrocytes or towards a neuronal linage.

In summary, as part of this thesis the Cor-1 cell line were introduced as a suitable model system to investigate eCB signalling. It was previously used to establish a solid basis for investigating the role of the eCB system in proliferation as well as migration. Here we used the Cor-1 cell line to establish the role of the eCB system in neuronal and astrocytic differentiation which proved to be not the case. Furthermore DAGLα expression levels were established during these differentiation

processes and we found, that a rapid reduction of DAGLα occurs during neuronal differentiation, while remaining level during astrocytic differentiation. The observed reduction occurs within 24 hours which lead us to be interested in what causes the decrease of DAGLα protein expression. In this context, a d-box motif, which can lead to degradation of the protein via the ubiquitin proteasome pathway, was detected within the sequence of DAGLα. No evidence was found supporting the hypothesis that the putative d-box causes the rapid reduction of DAGLα during neuronal differentiation. However, first evidence indicates that DAGL can be ubiquitinated. In order to be able to investigate DAGL regulation it is essential to measure enzyme activity and a new assay based on two surrogate substrates has been published. The chromogenic substrate was successfully adapted to measure DAGL activity in DAGL overexpressing Cor-1 cells and will be a useful tool to address further questions. Both DAGLα and DAGLβ can metabolise the surrogate substrate .

As mentioned above, a role for the eCB system in Cor-1 proliferation has been identified. It is not known, what the upstream driving mechanisms of the eCB system are and EGF, FGF and insulin were investigated as part of this thesis in this context. Microarray and other data indicate, that neither FGF nor EGF are upstream of the eCB system. A subset of transcripts appears to be regulated by the EGFR as well as the CB1/2 receptors, which could be explained by a common signalling node. mTOR was suspected to be this signaling node, but evidence was found supporting the notion that it is not the signalling node.

This thesis focuses on DAGL-dependent CB1/2 receptor signaling. As mentioned in the introduction there are non-CB1/2 receptors which are capable of interacting with the endocannabinoid. For that reason it is essential to investigate the roles of DAGL and the CB1/2 receptors on the investigated processes. This has been done for example when establishing the role of the eCB system in NSC proliferation (Goncalves et al., 2008) where DAGL as well as CB1/2 receptor antagonists were utilized. This approach does not exclude the possibility of non-CB1/2 receptors playing a role for NSC proliferation, but it does show a clear role of DAGL dependent CB1/2 receptor signalling in NSC proliferation. Both 2-AG and arachidonic acid have been investigated in NSCs as well as the brain and in many

contexts their effects remain difficult to separate (Alger and Kim, 2011; Alger, 2012). This is another reason why it is important to use a combination of CB1/2 receptor antagonists and DAGL inhibitors as using.

7.1. The Cor-1 cells as a model system to investigate eCB signaling

In the context of neurogenesis research, neurosphere assays have been widely used to carry out *in vitro* assays (Reynolds and Weiss, 1992) as well as being used to investigate the role of the eCB system in NSC proliferation (Molina-Holgado et al., 2007). In brief NSCs are plated at a low density and give rise to neurospheres. While being a useful tool, the disadvantage of neurosphere assays is that of the heterogeneous nature of the neurosphere containing NSCs as well as their progeny (Reynolds and Weiss, 1992; Reynolds and Rietze, 2005). Therefore there are limitations on researching individual signaling pathways in one of the cell types like the NSCs using this assay.

Another kind of *in vitro* assay is based on adherent NSC cultures and possibly one of their main advantages is their homogenous cell population. The Cor-1 cells were termed NSCs when they were generated and are similar to embryonic stem cells (Conti et al., 2005). Similar adherent rodent and human cell lines with comparable expression profiles and behaviour have been generated from foetal as well as adult tissue as well as ES cells (Conti et al., 2005; Sun et al., 2008). Cor-1 cells maintain certain aspects of "true" NSCs as they are tripotent and can be grown for an extended period of time without differentiation as well as expressing a range of NSC markers (Conti et al., 2005; Glaser et al., 2007).

In order to generate new neurons, NSC cells have to proliferate, migrate to their destination, and differentiate into functional cells. All these processes can be investigated using Cor-1 cell assays. DAGL is expressed in SVZ ependymal and proliferating cells and inhibiting DAGL or CB2 results in reduced proliferation of cultured NSCs (Goncalves et al., 2008). These findings were based on the DAGL inhibitors RHC80267 and THL. Since then the DAGL inhibitor OMDM-188 has become available to us, which has been shown to have a higher specificity (Ortar et

al., 2008). OMDM-188 was used in a Cor-1 cell based proliferation assay in this thesis and supports the importance of DAGL for NSC proliferation.

The eCB system has been demonstrated to be relevant for some cell differentiations. CB1 receptor activation can enhance progenitor proliferation as well as their differentiation into astroglial cells *in vitro.* Furthermore, the progenitor proliferation and astrogliogenesis are impaired in CB1 KO mice (Aguado et al., 2006). DAGL, 2-AG and MAGL have been shown to be expressed by oligodendrocytes throughout different developmental stages and DAGLα and DAGLβ were found in cultured oligodendrocyte progenitors. Furthermore, the DAGL inhibitor RHC80267 blocked the maturation of these cells and this process might be mediated via the ERK/MAPK pathway (Gomez et al., 2010). In the chick the CB1 expression pattern mirrors neuronal differentiation throughout the early developmental stages (Begbie et al., 2004) and the importance of eCB signaling for neuronal differentiation is discussed in considerable detail elsewhere (Keimpema et al., 2011). Given the role of eCB signaling for the proliferation and migration of Cor-1 cells, we wanted to establish, if the eCB system is relevant to Cor-1 cell differentiation. The Cor-1 cells can be differentiated into astrocytes, neurons and oligodendrocytes (Conti et al., 2005; Glaser et al., 2007) and we wanted to establish the role of eCB signaling for neuronal and astrocytic differentiation but no evidence to support this hypothesis was identified. While endogenous DAGLα expression is unchanged by astrocytic differentiation, a reduction in overall DAGLα levels was seen upon neuronal differentiation. At least part of the reduction is based on the rapid reduction of DAGLα in Tuj-1 positive interneuron. It has to be mentioned that no such reduction was seen in hippocampal interneurons (PhD thesis, Madeleine Oudin) and therefore might be limited to certain types of interneurons. Furthermore, Cor-1 cells have been used to research NSC migration elsewhere (PhD thesis Madeleine Oudin, Oudin et al., 2011b).

Cor-1 cell based *in vitro* findings showing a reduced proliferation when the CB2 receptor or DAGL were inhibited were mirrored by a drop in progenitor cell proliferation in young mice. Reduced progenitor proliferation in the SVZ furthermore led to a smaller number of neuroblasts migrating to the OB (Goncalves

et al., 2008). Using a Cor-1 cell based migration assay a role for eCB signaling in Cor-1 cell migration was uncovered. *In vivo* experiments confirmed a role of the eCB system for some aspects of neuroblast formation such as nucleokinesis and length and branching of migratory neuroblast processes (Oudin et al., 2011b). This highlights that the Cor-1 cells overall are an appropriate model system to investigate the eCB system and various aspects of neurogenesis. However, considering the importance of the NSC niche for NSCs it has to be stressed that all *in vitro* results need to be confirmed *in vivo*.

7.2. Regulation of DAGLα

The DAGL enzymes can be seen as the switch between DAG and 2-AG signaling. Since both molecules have important signaling roles it is reasonable to assume that DAGL activity is regulated. In the past the field of eCB research mainly focused on the processes "downstream" of 2-AG and/or anandamide interactions with the CB1/2 receptors. Relatively little focus was put onto the enzymes generating the eCBs and to date little is known about DAGL regulation (Reisenberg et al., 2012) but collective evidence from the literature points towards it and will be discussed in more detail below.

To understand the requirements for DAGL activity is to understand what initiates eCB signaling. To my knowledge, there is no other accurate way to monitor DAGL activity other than measuring 2-AG levels, which is a relatively technical, chromatography based method (Zoerner et al., 2012). Therefore it is crucial to establish quicker, higher throughput methods as this will help to investigate DAGL activity. A recent paper published two surrogate substrates for a membrane based DAGL assay (Pedicord et al., 2011), which was tested for its transferability to Cor-1 cells in this thesis. The surrogate substrate PNP was successfully established for use in Cor-1 cells. Further testing will be required to establish how comparable the surrogate substrate and DAG are, but overall this novel assay is a promising tool and will help to understand DAGL activity.

7.2.1. DAGL phosphorylation and structural features

The DAGLs belong to the α/β-hydrolase family with the signature core of an α/β-sheet consisting of 8 strands connected by helices. The described feature is located

in the catalytic domain of DAGL and contains the catalytic tirade. Bioinformatic analysis revealed a possible "lid" or "cap" loop, which potentially guards the hydrophobic catalytic cavity against water, but allows lipid access by opening upon membrane absorption (Miled et al., 2003; Reisenberg et al., 2012). There is cumulative mass spectrometry data pointing towards phosphorylation of the DAGLs within the catalytic domain and the regulatory loop (Wang et al., 2006; Rikova et al., 2007; Dephoure et al., 2008; Brill et al., 2009; Mayya et al., 2009; Huttlin et al., 2010; Hsu et al., 2011). Therefore it is reasonable to assume, that phosphorylation might be involved in DAGL activity (Reisenberg et al., 2012). Moreover, if the putative DAGL "lid" indeed regulates access to the catalytic cavity the substrate size and other properties are a crucial part of gaining access to the catalytic side.

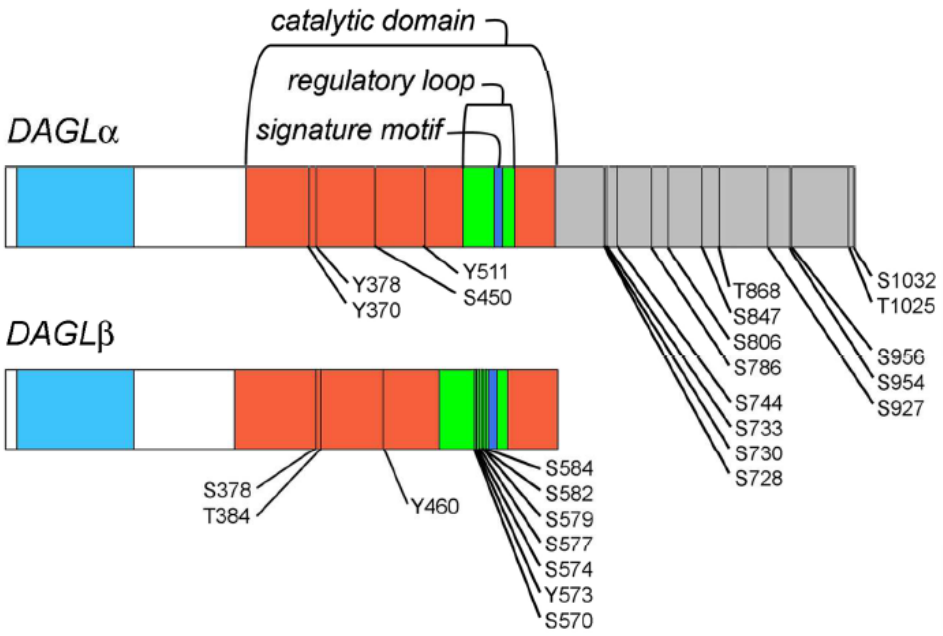


Figure 7.2: The DAGLα/β phospho map

The DAGLα/β phospho map is numbered according to the human enzyme and represent the currently known phosphorylation sites. The catalytic side of the DAGL enzymes is presented in red, the signature motif in blue and the regulatory loop is coloured in green. (Figure adapted from Reisenberg, Singh et al. 2012)

7.2.2. Ubiquitination and degradation

It is important to understand DAGL degradation as it might be an additional mechanism to transcriptional regulation to allow a tight regulation of DAGL. A "switch" in expression pattern is for example observed between development and the adult brain for the DAGLs and is discussed in more detail in the introduction of this thesis. While DAGLα is expressed in the axonal tracts during development in the adult it is highly enriched in dendrites (Bisogno et al., 2003). The degradation of DAGLα on the protein level -to the best of my knowledge-has not been investigated. Based on an observation in the context of neuronal differentiation of Cor-1 cells, we developed an interest in this process. In this report some preliminary results point towards ubiquitination of DAGLα in Cor-1 cells. Since the ubiquitination was seen in an overexpressing cell line it is possible that the ubiquitination is part of a regulatory mechanism to manage the higher than normal DAGLα protein levels or it might account for an increased mis-folding of the DAGLα enzyme in this context. While requiring further investigation this observation is still interesting as it is a potential regulatory mechanism. In the context of membrane proteins, ubiquitination has been shown to lead to degradation via the lysosome pathway (Piper and Luzio, 2007) and might be part of the mechanism to regulate DAGL levels.

7.2.3. Palmitoylation

S-Palmitoylation is a reversible and dynamic form of post translational modification, which is regulated through protein acyltransferase and protein acylthioesterases. It describes the linkage of a long -chain fatty acid to a cysteine residue of membrane protein and increases the hydrophobicity of the protein and enhances the membrane association. Especially, its reversibility makes it versatile and palmitoylation has been shown to be involved in a wide range of processes such as protein-protein interaction, subcellular trafficking and synapse plasticity (Smotrys and Linder, 2004; Resh, 2006). In the latter, it regulates a variety of aspects of neuronal protein trafficking (Fukata and Fukata, 2010). One prominent member of the lipase family that is known to be palmitoylated is Phospholipase D (PLD). Palmitoylation has been shown to be essential for its correct membrane localization and endocytosis (Du et al., 2003). It furthermore has been shown that a lack of

palmitoylation impairs mono-ubiquitination and thereby most likely its subcellular trafficking (Yin et al., 2010). DAGL has been identified in a neural palmitoyl-proteomics screen as a candidate protein for palmitoylation (Kang et al., 2008). A further indication for a post translational modification in general is a different molecular weight in different brain regions such as the hippocampus and cerebellum (unpublished observation and Yoshida et al., 2006). Moreover, DAGLα was identified to be localized in lipid rafts (Rimmerman et al., 2008), which are known to be an assembly point for palmitoylated proteins and often are involved in efficient signal transduction (Resh, 2006). Taken together these publications make palmitoylation of DAGL highly possible and it might be a potential process which is part of its regulation.

7.2.4. Transcriptional regulation of DAGL

The core promoter of mammalian DAGLα and its regulatory elements have been identified and an enhancer and a suppressor region were recognized. Utilizing deletion analysis, two promoting elements including a GC-box were found and shown to particularly be involved in promoting expression in NSCs. Out of the three candidate transcription factors (growth response element 1, zinc finger DNA-binding protein 89 and specific protein 1 (Sp1)) only the later was shown to bind to the GC-box. Differentiation of a NSC line towards a GABAergic neuronal phenotype showed a drastic down-regulation of DAGLα, which was mirrored by a down-regulation of SP1 (Walker et al., 2009). Despite these initial findings, the transcriptional regulation of DAGLα/β is relatively unexplored and requires further investigations.

7.3. The DAGLα signaling network

7.3.1. What drives eCB/DAGL signaling?

In order to understand DAGL/eCB signaling in NSCs, it is essential to understand what drives this signaling pathway. In neuronal growth cones the FGFR was demonstrated to be upstream of DAGL/eCB signaling (Williams et al., 2003) It has also been shown that EGF and FGF-2 are mitogens essential for the proliferation and maintenance of NSCs *in vitro* as well as *in vivo* and both mitogens are downregulated during aging (Enwere et al., 2004; Shetty et al., 2005). This coincides

with a reduced utilization of eCB signaling in older mice. While DAGL expression remains relatively unchanged in the SVZ of older animals, CB2 agonists can increase proliferation in these older mice, while having no fundamental effect on the same cells in younger animals (Goncalves et al., 2008). Therefore it could be hypothesized, that the reduction in eCB signaling in older animals might reflect a reduced upstream signaling stimulus. Comparing the transcriptional response of eCB signaling with that of EGFR or FGFR inhibition little correlation was seen between these signaling pathways. Additionally CB1 and/or CB2 receptor agonists were unable to recover reduced Cor-1 proliferation caused by a reduction in EGF concentration. These and other results indicate that neither EGF nor FGF2 signaling have a considerable influence on eCB signaling in Cor-1 cells.

Stem cells can react to metabolic fluctuations (Speder et al., 2011) and even though InsR and IGFR expression within the brain regions, such as the OB, hypothalamus and hippocampus was established in the late 70th (Havrankova et al., 1978; Unger et al., 1989), the role of insulin signaling was more recently linked to brain cell progenitor proliferation (Hodge et al., 2004; Popken et al., 2004; Lehtinen et al., 2011). Growth factor signaling, especially via the IGFR, and progenitor cell division were linked by different groups (Baker et al., 1993; Hodge et al., 2004; Popken et al., 2004; Liu et al., 2009). IGF-1 was implied to be involved in mammalian NSC division (Mairet-Coello et al., 2009) and identified to drive proliferation within the embryo (Joseph D'Ercole and Ye, 2008) as well as proliferation in the adult mammalian brain (Anderson et al., 2002). In this thesis I have shown that insulin withdrawal leads to a reduced Cor-1 cell proliferation, which can be partly recovered using CB1/2 receptor agonists. However CB1/2 receptor antagonists can further decrease Cor-1 cell proliferation indicating that while having an effect on eCB mediated proliferation, insulin cannot fully account for what is driving eCB signaling.

A so far not investigated candidate to drive eCB signaling is progesterone. Gonadal hormones have been linked to gliosis (Arevalo et al., 2012) and the eCB system is targeted by estradiol in the context of reactive gliosis regulation. CB1 and CB2 receptor antagonists moreover are capable of reducing the estradiol effect on

reactive astrocytes (Lopez Rodriguez et al., 2011) therefore establishing a link between gonadal hormones and eCB signaling. Progesterone also has been linked to the eCB system and while commonly being associated with women, it is also present in men. Progesterone is involved in maintenance of eCB protein levels and FAAH can be stimulated by progesterone and leptin in some cell lines (Gasperi et al., 2005; Karasu et al., 2011). It has also been reported that progesterone levels reduce with aging (Barron and Pike, 2012) allowing the possibility that this molecule may drive the eCB signaling pathway (see above). Progesterone is present n the Cor-1 cell growth media and it would be interesting to investigate a possible relationship between progesterone and eCB signaling in Cor-1 cells.

Moreover, Cor-1 cells do not proliferate well in low cell density conditions (unpublished observation), which indicates that the cells secrete certain messenger substances themselves, which might drive eCB signaling in Cor-1 cells.

7.3.2. Which signaling pathways are affected by DAGL activity?

In this thesis, DAGLα has been explored as part of the eCB pathway. The DAGLs however are in a prime location to influence more than one signaling pathway as they influence the steady state levels of different lipid messengers and via them a large number of other enzymes. In some species such as *Drosophila*, DAGL is expressed in the absence of an orthologue to the vertebrate CB receptors (Elphick and Egertova, 2005), which might suggest that they are relevant in non-eCB contexts.

Upon GPCR (G-protein coupled receptor) and/or RTK stimulation DAG is hydrolysed from phosphatidylinositol 4,5-bisphosphate (Brose et al., 2004). The DAGL enzymes then synthesis 2-AG from DAG; thereby influencing the levels of these molecules locally. DAG has a wide range of roles within the cell and it can act either as a substrate to DAGLα/β as discussed here or other enzymes such as DAG Kinase (DAGK). DAGK phosphorylates DAG to generate phosphatidic acid (PA) which might have signaling roles (Luo et al., 2004). Perhaps even more importantly, DAG regulates a multitude of enzymes most prominently by activation of the PKCs (Mellor and Parker, 1998). Therefore through these mechanisms alone, the DAGLs

indirectly influence an array of cellular activities such as cell growth and gene transcription via DAG (Mellor and Parker, 1998; Luo et al., 2004).

The DAGLs were first discovered in the context of arachidonic acid release from mast and platelet cells (Prescott and Majerus, 1983; Allen et al., 1992). In this context 2-AG is hydrolysed by MAGL to arachidonic acid, which is a second messenger molecule. Amongst other roles arachidonic acid serves as a precursor to produce eicosanoids and can be utilized by enzymes such as COX, which generate the prostanoid prostaglandin H_2 (Rapoport, 2008). In another context, arachidonic acid can be hyperoxidated by the liopoxygenases as part of the inflammatory response (Harman et al., 2004). Despite arachidonic acid levels being produced via different pathways such as via members of the phospholipase A2 family (Rapoport, 2008), the DAGL enzymes contribute significantly to the arachidonic acid levels via MAGL. The recently generated DAGL KO animals underline the importance of the DAGL/MAGL pathway for arachidonic acid steady state levels as has been demonstrated in DAGLα KO mice where an ~80% reduction in arachidonic acid levels was observed in the brain and spinal cord with a similar reduction in 2-AG levels. In adipose tissue however, despite a 50% reduction in 2-AG levels, the arachidonic acid levels remained unaltered (Gao et al., 2010) highlighting the importance of DAGL for arachidonic acid synthesis in the brain.

Therefore it always has to be kept in mind that while it is tempting to think about enzymes and signaling molecules in the context of a single signaling cascade, there is a vast amount of interactions and overlaps between signaling pathways. Furthermore the relationship with other signaling networks underlines that the regulation of the DAGL enzymes is essential to understand in more contexts than just in relation to eCB signaling.

7.3.3. The eCB system and what we still do not know

DAGLα expression and its' roles in various contexts is relatively well characterized, while little is known about DAGLβ. Studies relying on DAGL inhibitors like THL or RHC80267 do not differentiate between the two enzymes at all and therefore cannot be used to address this question. DAGLα/β KO studies revealed a greater

role for DAGLα in the brain, while DAGLβ seems to have a higher relevance to the liver (Gao et al., 2010). Further studies in these KO animals should prove to be a potent tool to further investigate the differences between the DAGL enzymes (Gao et al., 2010; Tanimura et al., 2010). Considering the many roles of DAGL signaling, it is somewhat surprising that the DAGL KO animals are relatively normal. To what extend can the DAGLs stand in for one another? The generation of double DAGLα/β KO mice might help to address this question.

It furthermore is unclear how the eCBs are released and/or taken up by cells. Is this an active process? Are some of the enzymes which are part of the eCB system involved in this? The eCBs are thought to be produced "on demand" (Marsicano et al., 2003), and unlike peptide messengers it is not thought to be possible to store these lipophilic molecules in vesicles. Considering the lack of information on these topics it could be proposed, that the eCB also could be produced and released "on demand" rather than being produced on demand (Alger, 2012).

It is the centre of many discussions to what extend anandamide or 2-AG mediated certain processes and this thesis focused very much on DAGL/2-AG mediate eCB signaling. The DAGL KO animals were thought to be able to give a final answer to the 2-AG vs. anandamide related questions. However anandamide levels were significantly changed in the brain of DAGLα KO mice and a trend was seen in DAGLβ KO mice (Gao et al., 2010). This highlights that the eCB system is interwoven to a high level and careful studies have to be taken to further separate 2-AG and anandamide functions.

7.4. Future directions

In order to further understand eCB signaling it is necessary to understand when and where DAGL synthesizes 2-AG. Most research in the field of the DAGL enzymes currently does not distinguish between expression of the enzymes and their activity. To investigate this further it is essential to have appropriate methods to assess DAGL activity. While it is possible to measure 2-AG levels with a combination of chromatographic techniques, this requires the suitable equipment, expertise and is relatively time consuming. Furthermore such a technique does not distinguish

between DAGLα and β activity. A surrogate DAGLα substrate assay has been published recently (Pedicord et al., 2011) and one of the two published substrates was successfully adapted to the Cor-1 cell line. Despite requiring further research to comparable the native and the surrogate substrate, this is a highly promising new tool. In the context of DAGL phosphorylation it might help to determine the effect of different phosphorylation events on DAGL activity and moreover might help to identify which kinases are involved in this process. In relation to the question what is driving DAGL dependent eCB signaling it will allow a quicker form of screening potential candidates.

Much accumulative evidence points towards DAGL being phosphorylated and this phosphorylation might be associated with enzyme activity (Reisenberg et al., 2012). It would be immensely interesting to explore this further, as phosphorylation almost always translates into a change of enzymatic activity. While identifying phosphorylation sites is far from trivial (Mann, Ong et al. 2002; Rigbolt and Blagoev 2012) it would be a considerable step towards understanding DAGL activity and experiments exploring DAGL phosphorylation are currently undertaken by our lab. Moreover, several phosphorylation sites have been predicted (Reisenberg et al., 2012) and 5 sites were selected for further analysis by our lab. Phospho-DAGLα antibodies were generated, but more experiments are needed to determine the phospho specific nature of these antibodies as well as establishing connections to possible roles of these potential phosphorylation events. In summary, two tools to establish DAGL activity are currently being investigated by our lab potentially holding the key to establishing DAGL signaling and regulation.

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